Transplantation of interleukin-10-expressing human neural stem/progenitor cells for hypoxic-ischemic brain injury in neonatal mice

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Transplantation of interleukin-10-expressing human neural stem/progenitor cells for hypoxic-ischemic brain injury in neonatal mice

Directed by Professor Kook In Park

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ABBREVIATION LIST

CNS	Central nervous system
DNA	Deoxyribonucleic acid
EdU	5-ethynyl-2′-deoxyuridine
GFP	Green fluorescent protein
HI	Hypoxic-ischemic
hNSPC	Human neural stem/progenitor cell
IL	Interleukin
PCR	Polymerase chain reaction
RNA	Ribonucleic acid

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Transplantation of interleukin-10-expressing human neural stem/progenitor cells for hypoxic-ischemic brain injury in neonatal mice

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Neonatal hypoxic-ischemic brain injury, a major cause of neonatal death and serious long-term neurological disability, results in extensive loss of cerebral parenchyma, neural cells, and connection. Although promising neuroprotective strategies have been studied, current management of HI brain injury has been limited supportive measures. The brain is a major target for inflammatory mediator actions and that inflammation or cytokines could be neurotoxic with direct effects on nerve cells. Although the mechanisms involved in inflammation-induced early brain injury are not clearly understood, recent evidence has shown that inflammatory responses in the fetus and neonate can contribute towards inflammatory cerebral white matter damage.

Neural stem/progenitor cells are able to continuously self-renew and give rise to both neuronal and glial lineages. Upon implantation into an HI-injured brain, multipotent NSPCs not only engraft, migrate toward lesion sites and differentiate into neural cell types lost to injury, but also provide trophic/immunomodulatory factors, all of which are promising therapeutic options for neural repair.

Interleukin-10 is a typical anti-inflammatory cytokine that limits innate as well as adaptive immune

responses protecting the host from immune-mediated tissue damage. In a variety of different cell types, IL-10 not only mediates down-regulation of a broad spectrum of proinflammatory mediators such as IL-1, IL-6, IL-12, IFN-γ, and TNF-α, but also enhances alternatively activated microglia, thereby promoting tissue repair and remodeling.

In this study, we investigated the regenerative ability and action mechanisms of IL-10-transduced human NSPCs (IL-10-NSPCs) using a lentiviral vector implanted into the HI-damaged brain to develop a novel human NSPC-based gene therapy for neonatal HI brain injury. IL-10-NSPC showed increased proliferation, promoted neuronal differentiation over glial differentiation, anti-inflammatory effect on immune cells (microglia and macrophage), and changed polarization of microglia and macrophage from M1 to M2 type both *in vitro* and *in vivo*. Implanted IL-10-NSPCs reduced cerebral infarction volume, facilitated neurobehavioral recovery, reduced proinflammatory cytokines levels in the HI-injured brain, modulated inflammation through the polarization change of microglia and macrophage into anti-inflammatory activation, and exhibited neuroprotection and angiogenesis by secretion of growth and angiogenic factors *in vivo*.

Based on these results, we postulate that IL-10-expressing human NSPCs facilitate functional recovery and neuroprotection after neonatal HI brain injury through synergistically enhanced anti-inflammatory/immunomodulatory properties, neuronal survival and angiogenesis.

Key words: neonatal hypoxia-ischemia brain injury, human neural stem/precursor cells, interleukin-10, transplantation, inflammation

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I. INTRODUCTION

Hypoxic-ischemic brain injury in newborn infants is a major cause of mental retardation, cerebral palsy, epilepsy, learning disabilities, and even death. Perinatal HI brain injury occurs in 2–4 per 1000 live term births, and approximately 60% of preterm HI infants suffer from permanent brain damages, although therapeutic hypothermia has been shown to improve recovery.[1] The pathogenesis of the ischemic brain is not described only by the pathway of hypoxic cell death, but reflects continuous changes in genetic expression and levels of intrinsic oxidant radicals, growth factors, and inflammatory mediators such as cytokines, adhesion molecules, etc.[2-4] Two phases of HI-induced neuronal death have been identified in both clinical and experimental studies.[5,6] The immediate phase, primary neuronal death, is related to cellular hypoxia with exhaustion of the cell's high-energy stores (primary energy failure). The second phase, delayed neuronal death,[7] occurs after a latent period of at least 6 hr, and is associated with encephalopathy and increased seizure activity. Delayed neuronal death accounts for a significant proportion of final cell loss even after very severe insults. The mechanisms involved in delayed neuronal

death include excitotoxicity, apoptosis and microglial activation.[8]

Microglia are the resident immune cells in the brain and play an essential role in central nervous system development, maintenance and repair.[9] However, altered microglial activity is associated with common human diseases, such as migraine, stroke, dementia, traumatic injury, epilepsy and Parkinson's disease.[10-12] Microglia and macrophages are among the most potent modulators of CNS repair and regeneration;[13] however, these cells are apparently double-edged swords in neurological recovery. On the one hand, activated microglia and macrophages promote brain recovery by clearing cell debris, resolving local inflammation and releasing trophic factors.[13-16] On the other hand, these cells can hinder CNS repair and expand tissue damage.[15,17,18] *In vitro*, classically activated (M1) microglia and macrophages release destructive proinflammatory mediators. In contrast, alternatively activated (M2) microglia and macrophages clear cellular debris through phagocytosis and release numerous protective and trophic factors.[19,20]

Regeneration of the CNS was previously considered impossible, despite the observation that focal cerebral ischemia promotes neurogenesis of endogenous neural stem/progenitor cells (NSPCs) in the subventricular zone of the lateral ventricles and subgranular zone of the dentate gyrus and induces their migration towards the ischemic boundary.[21] Several studies have suggested that transplantation of exogenous NSPCs may have beneficial effects on the outcome following HI brain injury;[22-24] however, the underlying recovery mechanisms are unknown.

Stem cell therapies have been attempted with various stem/progenitor cell preparations, including mesenchymal stem cells, multipotent astrocytic stem cells, and human umbilical cord blood cells.[25] Among these, NSPCs show more restricted neural differentiation capabilities committed to specific subpopulation lineages. NSPCs are derived from specific spatiotemporal neural tissues or are generated from embryonic and/or induced pluripotent stem cells.[26,27] They have self-renewal capability, and can give rise to neurons, astrocytes, and oligodendrocytes.[28,29] Human NSPCs (hNPSCs) can also be derived from the developing human fetal brain between 8-20 wk of gestation.[30,31] Multipotent NSPCs derived from the

developing or adult brain can be cultured and grown in the presence of mitogens, such as epidermal growth factor and fibroblast growth factor-2 either as monolayers or as free floating spherical aggregates (neurospheres).[30-33] When NSPCs are implanted into a diseased or injured nervous system, they show not only preferential extensive migration to and engraftment within areas of discrete abnormalities but also the capability to replace diseased tissue appropriately (cell replacement effect).[34-36] Apart from replacing lost cells, NSPC based therapy can also provide a regenerative microenvironment for other cells residing in diseased brains (bystander effect).[23,37-39] Early studies aimed to establish whether exogenous NSPCs could engraft and replace dying cells, and many showed that the donor cells survived and migrated toward the injury site. However, the effect derived from the net increase in cell numbers was generally negligible.[25] Notably, the transplanted NSPCs were usually maintained with little differentiation, and it is not clear if these treatments resulted in fully functional and integrated neurons or glia.[23,40,41]

These findings suggest that the key mechanisms leading to therapeutic effects may be the release of neurotrophic and immunomodulatory/immunosuppressive factors.[25,42] In the case of neonatal HI brain injury, stem cell treatment at different time points during the evolution of injury may allow a range of potentially complementary mechanisms to be targeted.

NSPC transplantation is a promising tool for regeneration of damaged brain in various neurological disorders,[43] and has been investigated as a means to replace lost neurons and reconstitute lost brain tissue. Researchers have been eager to find efficient methods to transplant NSPCs into the brain and differentiate them into functional neurons. However, a number of studies have shown that NSPCs transplantation rarely results in a significant number of terminally differentiated neurons, although the transplantation induces significant functional recovery of the animal models. This raises the suspicion that the beneficial effect of NSPCs in disease models may be also attributable to alternative biologic properties, such as that transplanted NSPCs may attenuate deleterious inflammation, protect the CNS from degeneration, and enhance endogenous recovery processes.[44] Several researchers have proposed that NSPCs transplantation may protect the CNS from inflammatory damage via an indirect mechanism rather than by direct cell

replacement.[23] NSPCs rescue degenerating neurons by modulating the host environment by exert immune-like functions to induce apoptosis of encephalitogenic T cells,[38,45] and suppress peripheral adaptive immune responses.[46]

Interleukin-10 is the most important cytokine with anti-inflammatory properties. It is produced by activated immune cells, in particular monocytes/macrophages and T cell subsets including regulatory T cells and T helper 1 cells. IL-10 acts through a transmembrane receptor complex, composed of IL-10 receptor 1 and receptor 2, and regulates the function of many different immune cells, influencing several functions of the microglia and monocytes/macrophages that are responsible for a positive role of these cells in both innate and specific immunity, such as release of immune mediators, antigen presentation, and phagocytosis. At the same time, it enhances the inhibitory, tolerance inducing, and scavenger functions of these cells. IL-10 also inhibits release of proinflammatory mediators from microglia and monocytes/macrophages and therefore inhibits LPS- and IFN-g-induced secretion of TNF-α, IL-1b, IL-6, IL-8, G-CSF, and GM-CSF.[47,48]

Several lines of evidence support that the cerebral expression of IL-10 is beneficial after experimental stroke, as transgenic mice overexpressing IL-10 in astrocytes, microglia, and endothelial brain cells showed smaller infarcts.[49] IL-10 gene transfer using adenoviral vectors also reduced infarct volume in a model of photothrombotic ischemia in rats.[50] Mice deficient in IL-10 showed larger infarctions after permanent middle cerebral artery occlusion.[51] Animals deficient in IL-10 lack the anti-inflammatory drive of this cytokine, and therefore provide a good model to test whether and how IL-10 is involved in stroke outcomes. Consistent with these findings, low levels of circulating IL-10 in patients with lacunar stroke was associated with a worse outcome.[52]

In this study, we investigated the regenerative ability and action mechanisms of IL-10-transduced human NSPCs (IL-10-hNSPCs) implanted into the HI-injured brain to develop a novel hNSPC-based gene therapy for neonatal HI brain injury.

II. MATERIALS AND METHODS

1. Human neural stem/progenitor cell culture

Human fetal tissue from a therapeutic abortus at 13 wk of gestation was obtained with full parental consent and the approval of the research ethics committee of Yonsei University College of Medicine, Seoul, Korea. The methods of acquisition conformed to NIH and Korean Government guidelines. The telencephalic region of CNS tissue was freshly dissected, dissociated in trypsin (0.1% for 30 min), and seeded into tissue culture-treated 100-mm plates (Corning, Tewksbury, MA, USA) at a density of 200,000 cells/ml of serum-free growth medium, which consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (Gibco, Grand Island, NY, USA) supplemented with penicillin/streptomycin (1% vol/vol; GIBCO) and N2 formulation (1% vol/vol; GIBCO). Mitogenic stimulation was achieved by adding 20 ng/ml fibroblast growth factor-2 (FGF-2; Minneapolis, MN, USA) and 10 ng/ml leukemia inhibitory factor (Sigma, St Louis, MO, USA). Heparin (8 μg/ml; Sigma) was added to stabilize FGF-2 activity. All cultures were maintained in a humidified incubator at 37°C and 5% CO_2 in air, and half of the growth medium was replenished every 3-4 days. Passage of these cells was undertaken every 7-8 days by the dissociation of bulk neurospheres with 0.05% trypsin/EDTA (T/E; GIBCO).

2. Viral vector construction and preparation of IL-10-expressing human neural stem/progenitor cells

A second-generation of lentiviral vector containing EF-1α promoter was obtained from Trono lab (Prof. Didier Trono). We constructed recombinant lentiviral vectors bearing human IL-10 and green fluorescent protein (GFP) controlled by an internal ribosome entry site (IRES). The lentiviral vectors were produced by co-transfection of a transfer (pWPI-human IL-10-IRES-GFP), packaging (psPAX2) and envelope (pMD2.G) vector into 293FT cells (Thermo Fisher Scientific, Rockford, IL, USA) by calcium phosphate

transfection method. The infectious recombinant virus was titrated on HeLa cells by FACS (BD biosciences, San Jose, CA, USA). Lv-IL-10-IRES-GFP-infected hNSPCs were seeded onto cell culture dishes with 4-fold multiplicity of infection (MOI). IL-10 expression was checked by reverse transcriptase polymerase chain reaction (RT-PCR) and western blot.

3. Induction of experimental focal hypoxic-ischemic brain injury and cell transplantation

We used CD-1 mice and induced an HI injury. The right common carotid artery of anesthetized mice at postnatal day 7 was ligated with 6-0 surgical silk. The incision was closed, and the animals were kept warm (37–38°C) until awake, then returned to their dams for 1.5–2 hr. The stabilized mice were placed in an acrylic chamber with a hypoxic atmosphere (8% O_2 and 92% N_2)and a 39°C heating pad for 1.5 hr. The animals recovered in room air and were returned to their dams. All mice received the same care and housing, and were evenly distributed between control and treatment groups. On day 3 after HI injury (postnatal day 10), the pups were anesthetized, and an incision was made through the dorsal midline of the scalp to inject IL-10-expressing hNSPCs (IL-10-hNSPCs or IL10-NSC group; 10 μl cell suspension at 8×10^4 cells/μl), GFP-expressing hNSPCs (GFP-hNSPCs or GFP-NSC group), or vehicle into the HI-injured site of each mouse brain with a glass micropipette (0.3 mm diameter). Cyclosporine (10 mg/kg) was intraperitoneally administered daily beginning a day before surgery until sacrifice. The procedures were approved by the Animal Care and Use Committees of Yonsei University College of Medicine (Seoul, Korea).

4. *In vitro* differentiation studies of IL-10-expressing human neural stem/progenitor cells

To identify IL-10-expressing hNSPCs, we stained IL-10-expressing hNSPCs (IL-10-hNSPCs), GFP-expressing hNSPCs (GFP-hNSPCs), and compared the cell fates. IL-10-hNSPCs or GFP-hNSPCs was

trypsinized and plated on poly-L-lysine (10 μg/ml; Sigma)-coated eight-well chamber slides (Nunc) at $8x10^4$cells/well density and these two distinct hNSPCs were differentiated for 5 days. The cells were fixed with 4% paraformaldehyde (PFA) in 0.1 M PIPES buffer (Sigma) for 10 min, rinsed three times with phosphate-buffered saline (PBS), and treated as described below. The fixed cells were blocked with 3% bovine serum albumin (BSA) and 10% normal horse serum with 0.2% Triton X-100 and incubated with the following primary antibodies: human nestin (1:200; Millipore, Billerica, MA, USA); GFAP (1:1000; DAKO, Glostrup, Denmark); STEM123 (1:500; StemCell INC); β-tubulin III (Tuj1, 1:500; Covance, Princeton, NJ, USA); Olig2 (1:500; Millipore); galactocerebroside (GalC, β 1:100; Sigma); Following rinsing in PBS, the cultures were incubated with species-specific secondary antibodies conjugated with fluorescein (Vector, 1:180) or Texas Red (Vector, 1:180), and DAPI (4', 6'-diamidino-2-phenylindole, Vector) was used as the nuclear stain. After immunofluorescence staining, the percentage of immunoreactive cells was evaluated using a fluorescence microscope (Olympus Optical Co. Tokyo, Japan). For each group, we counted cells in 10 fields that included 100–500 cells. The total number of cells was evaluated by counting DAPI-positive nuclei and marker-positive cells.

To obtain total RNA of IL-10-hNSPCs or GFP-hNSPCs, hNSPCs were trypsinized and plated on poly-L-lysine (10 μg/ml; Sigma)-coated 6-cm dish (Corning) at 1 x 10^6cells/dish and these two distinct hNSPCs were differentiated for 3 days to evaluate the gene expression of nestin, GFAP, Tuj1 and GalC. The differentiated hNSPCs were washed with PBS and resuspended in TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH, USA).

5. 5-ethynyl-2′-deoxyuridine proliferation assay

Cell proliferation was estimated by 5-ethynyl-2'-deoxyuridine (EdU) incorporation into newly synthesized DNA using Click-iT® Plus EdU Proliferation Kits for imaging and flow cytometry (Thermo Fisher Scientific) according to manufacturer's instructions. Human NSPCs were seeded onto 6-well plates

at 1 × 10^6 cells per well in N2 media supplemented with 20 ng/ml fibroblast growth factor-2 (FGF-2; R&D), 10 ng/ml leukemia inhibitory factor (Sigma), heparin (8 μg/ml; Sigma), and EdU (2uM); and incubated for 24 hr. EdU treated hNSPCs were dissociated as single cells, fixed in 4% PFA in 0.1M PIPES solution, permeabilized using Perm/Wash Buffer (BD Biosciences, Cat #554723) and labeled with Alexa Fluor 647 Azide (Thermo Fisher Scientific). The percentage of cells incorporated with EdU was analyzed by flow cytometry.

6. Oxygen-glucose deprivation

SH-SY5Y cells were dissociated and seeded on collagen (25 μg/cm^2; Gibco) coated 24-well plates at 80,000 cells/1 ml of 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 supplemented with 10 μM of retinoic acid (Sigma), then incubated for 5 days to differentiate neuronal cells. At 5 days after cell seeding, differentiated SH-SY5Y were indirectly co-cultured with 2×10^5 hNSPCs or fibroblast using Transwell with 0.4 μm pore insert (Corning) and incubated in an anaerobic chamber for 4 hr in Dulbecco's Modified Eagle Medium (DMEM, high glucose. Gibco; Cat #11965084) or DMEM (no glucose, Gibco; Cat #11966025).

7. Lactate dehydrogenase assay

Cytotoxic effects were measured using the Cytotoxicity Detection KitPLUS (LDH, Roche Diagnostics Ltd., Rotkreuz, Switzerland) to quantify the release of LDH from SH-SY5Y cells incubated in oxygen-glucose deprivation (OGD) conditions, according to the manufacturer's instructions. All the samples were conducted in triplicate.

8. BV2 cell culture

BV2 cells (murine microglia) were maintained in DMEM supplemented with 5% FBS and penicillin/streptomycin (1% vol/vol; GIBCO). Cells were seeded on 100-mm dish at 1×10^6 cells/10 ml culture media and split with fresh media daily. For stimulation, BV2 cells were treated with 100 ng/ml of lipopolysaccharide (LPS) for 24 hr and total RNA was extracted using TRI Reagent.

9. Isolation and culture of bone marrow derived macrophage

Femurs and tibias were isolated from mice. Both ends of the bones were cut with scissors and then flushed with 3–5 ml of 2% FBS in cold PBS with a 21G needle. Isolated marrow was passed through a 21G needle 4–6 times to dissociate the cells, and then the dissociated cells were passed through a 70 um cell strainer to remove cell clumps, bone, hair, and other cells/tissues. The cells were incubated with 0.8% of NH4Cl solution in ice for 10 min to remove red blood cells, and were centrifuged at 500 g for 5 min at 4°C. Cells were seeded at 1×107 bone marrow cells in 10 ml of bone marrow derived macrophage (BMDM) growth media (10% FBS and 1x P/S in IMDM, Gibco, 12440-053) on 10-cm ultra-low culture dishes (Corning) supplemented with 10 ng/ml of M-CSF, then incubated for 7 days.

10. Preparation and treatment of conditioned media

IL-10-hNSPCs or GFP-hNSPCs were seeded at 1.2×10^7 cells/12 ml of N2 media on 100-mm culture dishes and incubated for 3 days. Media were then harvested and cleared by centrifugation at 3,000 g for 5 min. The conditioned media (CM) was divided into aliquots and stored at 80˚C until use. To evaluate effects of the CM, BV2 cells were seeded at 2×10^6 cells/3 ml of culture media and incubated for 1 hr. Old media were then replaced with 100 ng/ml of LPS and CM. After a further 24 hr incubation, the BV2

cells were washed and collected in Tri Reagent for RNA.

11. Migration assay for chemotaxis

To determine the migration of BV2 or THP-1 to the hNSPCs, Lv-IL-10-hNSPCs, we used CytoSelect™ 24-well cell migration assay (5 μm, Fluorometric Format) (Cell Biolabs, Inc.), according to the manufacturer's instructions. Singly dissociated hNSPCs or fibroblast were seeded onto poly-L-lysine-coated 24-well plates (lower chamber) at 500,000 cells/ml of 1:1 mixture of DMEM and Ham's F12, then incubated for 24 hours. BV2 cells (50,000 cells/100 ul of the same media) were added into the upper chamber of the 5.0 mm porous Transwell and incubated for 4 hr in a cell culture incubator (upper chamber). Migratory cells attached to the bottom side of the Transwell were dissociated from the membrane by the addition of cell detachment buffer to the lower chamber. Detached cells were lysed and quantified using CyQuant® GR fluorescent dye and the FlexStation®3 Reader.

Migration of endothelial progenitor cells (EPCs) was assayed using a disposable 96well chemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD). Briefly, EPCs were harvested with 0.05% trypsin containing 0.02% EDTA, washed once, and suspended in endothelial cell basal medium-2 (EBM-2) at a density of 2×10^5 cells/ml. A membrane filter with 8 μm pores of the chemotaxis chamber was precoated overnight with 20 μg/ml rat-tail collagen at 4 °C, an aliquot (100 μL) of EPCs suspension was loaded into the upper chamber, and CM of GFP-hNSPCs or IL-10-hNSPCs was then placed in the lower chamber. After incubation of the cells for 12 hr at 37 °C, the filters were disassembled, and the upper surface of each filter was scraped free of cells by wiping it with a cotton swab. The number of cells that had migrated to the lower surface of each filter was determined by counting the cells in four locations under microscopy at ×100 magnification after staining with hematoxylin and eosin.

12. Immunohistochemistry

The animals were anesthetized and transcardially perfused with cold PBS followed by cold 4% PFA in 0.1 M PIPES solution. The brain was carefully extracted, fixed overnight in 4% PFA in 0.1 M PIPES solution at 4°C, and subsequently transferred to 30% sucrose solution at 4°C until embedding. Optimal cutting temperature compound was used to embed the brains, and 16 μm sections were cut on a freezing cryostat and stored at 20°C. For fluorescence immunohistochemistry, sections were first blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 3% BSA in PBS with 0.3% Triton X-100. Primary antibodies were incubated overnight at 4°C. Appropriate secondary antibodies were applied for 70 min at 37°C followed by three PBS washes before they were mounted. Primary antibodies included human nuclei (hNuc, 1:100; Millipore), Ku80 (1:200; Cell Signaling tech, Danvers, MA, USA), STEM101 (1:100; StemCell INC, Newark, CA, USA), STEM121 (1:500; StemCell INC), and human IL-10 (1:100; R&D systems) for tracing hNSPCs and anti-human nestin (1:200; Millipore, Billerica, MA, USA); Tuj1 (1:500; Covance, Princeton, NJ, USA), GFAP (1:1000; DAKO, Glostrup, Denmark), STEM123 (1:500; StemCell INC), and Olig2 (1:500; Millipore) for identifying hNSPCs differentiation patterns and anti-ionized calcium-binding adapter molecule 1 (Iba-1, 1:500; Wako, Osaka, Japan); and CD68 (1:100; BD Biosciences) for microglia. Secondary antibodies were conjugated to fluorescein, or Texas Red (Jackson ImmunoResearch Laboratories, Inc). Immunolabeled cells were observed with a fluorescence microscope (Olympus), and a Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany) was used to acquire Z-stacks to assess merged images.

To investigate the implanted hNSPC differentiation profile, 100 or more human nuclear antigen, STEM121 or GFP positive cells in the peri-infarct area were scored for: nestin as a marker of immature NSCs, GFAP and STEM123 as markers of astrocytes, Tuj1 as a marker of immature and mature neurons, and Olig2 as a marker of oligodendrocyte progenitors at 2 wk post transplantation (n=4–5 per group).

13. Behavior test

Neurological severity score and cylinder tests were performed at 1, 2, 3, and 4 wk post transplantation in the following four groups of mice. We analyzed n=11–31 animals per group. The neurological functions of all animals were evaluated using the following five reflexes, with each exam scored as '0' if the response was normal and '1' if the animal showed an abnormal reflex:

(i) When the mouse was suspended by the tail above the ground (abnormal postures include rotating the body [torso twisting], and limb flexing [forelimb flexion]);

(ii) When the mouse was placed on its side, it immediately turned over to rest in the normal position with all four feet on the ground (right reflection);

(iii) When the dorsum of the paw contacted the edge of the table, the mouse immediately placed their paw on the surface (placing reaction); and

(iv) the mouse was placed on a board, and if the board was suddenly bounced, the mouse spread its toes (toe spreading).

In the cylinder test, all mice were located in an acrylic cylinder (10 cm diameter, 30 cm high) and recorded for 5 min. The video was used to evaluate how many times the mice placed their forelimbs on the cylinder walls, and limb use asymmetry (LUA) ratio was calculated as LUA (%) = [(non-impaired forelimb) – (impaired forelimb)] / [(non-impaired forelimb) + (impaired forelimb)] * 100. All tests were performed as a double blind.

14. Infarct volume measurement

We estimated lesion size as a percentage of the whole brain by using the following formula: [(area of contralateral hemisphere) - (area of remaining ipsilateral hemisphere)/(area of contralateral hemisphere) *

100]. The area of both hemispheres was measured in eight serial coronal sections per brain (200 μm apart) stained with Hematoxylin Gill's formula (Vector, Burlingame, CA, USA) and Eosin-Y (Sigma, St. Louis, MO, USA) using Virtual Microscope (Olympus BX51, Tokyo, JAPAN) and Image J (Broken symmetry software, NIH). The area of the infarct was averaged over the eight sections per animal and expressed as a percentage of total pixels within the microscope field. We analyzed n=7–11 animals per group at 4 wk post transplantation.

15. Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

The cryopreserved brain sections were fixed with 4% PFA in PBS for 20 min at RT and washed with PBS. The slides were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate buffer for 2 min at 4°C. After three washes, the sections were treated with Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture (Roche Applied Science) and incubated for 1 hr at 37°C. TUNEL positive nuclei at 4 wk post transplantation were microscopically analyzed.

16. Isolation of microglia and infiltrating immune cells from the brain

Brain mononuclear cells were isolated using a standard protocol described in detail previously.[53,54] Briefly, mice were perfused with cold PBS, the ipsilateral hemisphere was removed, diced and mechanically digested with Dounce homogenizer (Corning) in 7ml of RPMI 1640. Three ml of isotonic Percoll solution (Amersham) were added in the cell suspension to make a final 30% of Percoll. The cell suspension was gently laid on the surface of 70% Percoll solution and centrifuged (550 g, 30 min, 18°C). The cells in the interface between 30% and 70% of Percoll solution consisted of mononuclear cells, which were washed prior to experiments. The isolated cells were stained with CD11b antibody-conjugated with PerCP-Cy5.5 dye and CD45 antibody-conjugated with PE dye and were analyzed by LSRII (BD

Biosciences) flow cytometry to distinguish microglia and infiltrating macrophages from other immune cells.

17. Isolation of CD11b-positive cells from the brain

CD11b-positive cells in the injured brain, consisting of microglia and infiltrating macrophages, were isolated from the ipsilateral hemisphere of injured mice. Briefly, after perfusion with ice-cold PBS, brains were dissected, weighed, and enzymatically digested using the Neural Tissue Dissociation Kit P (Miltenyi Biotec, Germany) for 35 min at 37°C. Further processing was performed at 4°C. Tissue debris was removed by passing the cell suspension through a 40 μm cell strainer. After myelin removal (see below), cells were stained with CD11b antibody conjugated with magnetic beads (Miltenyi Biotec, Germany) in IMag™ buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA) for 15 min. CD11b+ cells were separated in a magnetic field using MS columns (Miltenyi Biotec, Germany). The amounts of antibodies and magnetic beads were calculated based on the number of cells obtained after myelin removal, using the manufacturer's guidelines. The CD11b-positive (bound on the column) and negative (effluent) fractions were collected and used for further analyses.

18. Area occupied by Iba-1- or CD68-immunoreactive microglia of the injured brain

Quantification of the area occupied by microglia was performed using Virtual Microscope (Olympus BX51, Tokyo, JAPAN) and Image J (Broken symmetry software, NIH). Images of Iba-1 immunoreactivity from cerebral cortical and penumbra regions were captured by Virtual Microscope, and the area occupied by Iba-1 (for total microglia) or CD68 (for activated microglia) immunoreactivity was determined using Image J by measuring number of pixels above a set threshold value and expressed as a percentage of total pixels within the microscope field. We analyzed n=7–11 per group at 4 wk post-

transplantation.

19. Complementary DNA synthesis, RT-PCR and quantitative Real-time PCR

One μg of total RNA isolated from samples was reverse-transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to manufacturer's instructions.

For reverse transcription PCR (RT-PCR), cDNA was amplified using Go-Taq polymerase (Promega, Madison, WI, USA) in a thermal cycler (Eppendorf, Happauge, NY, USA) according to the manufacturer's instructions. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. As a negative control, the extracted RNA was amplified with PCR without prior reverse transcription. The expression of PCR products was normalized relative to the expression of GAPDH.

Quantitative real-time PCR was performed in a total volume of 10 μl containing 5 μl of LightCycler® 480 SYBR Green I Master (Roche Diagnostics Ltd., Rotkreuz, Switzerland), 0.5 μM of each primer and 50 ng of synthesized cDNA using a LightCycler® 480 instrument (Roche Diagnostics Ltd). The cycling conditions were: 95° C for 5 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. All the samples were performed in triplicate.

The expression levels of each mRNA expression were normalized to the housekeeping gene GAPDH or 18S-rRNA using LightCycler® 480 Software, Version 1.5 (Roche Diagnostics Ltd). Primer sequences were retrieved from the PrimerBank Database (http://pga.mgh.harvard.edu/primerbank/).[55] Primer sequences are shown in Table 1.

Table 1. Sequences of primers for reverse transcription and real-time PCR

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')	
Il1b	TCCTGTGTAATGAAAGACGGCACA	CCCAGGAAGACAGGCTTGTGC	RT-

Tnfa	CGGTCCCCAAAGGGATGAGAA	CCTTGAAGAGAACCTGGGAGTAGACA	PCR
Il6	ATCTGAAACTTCCAGAGATACAAAG	GAAGATATGAATTAGAGTTTCTGTATCTC	
Inos	TTCAGCTCACCTTCGAGGGCA	CGTCTCGTCCGTGGCAAAGC	
IL10	CTAACATGCTTCGAGATCTCCGAGA	TCAAACTCACTCATGGCTTTGTAGATG	
Gapdh	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	
GAPDH	GAGAAGTATGACAACAGCCTCAAGATCA	CCACCACTGACACGTTGGCA	
Il1b	TTCAGGCAGGCAGTATCACTC	GAAGGTCCACGGGAAAGACAC	Real-time PCR
Tnfa	CCAGTGTGGGAAGCTGTCTT	AAGCAAAAGAGGAGGCAACA	
Il6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC	
Inos	GGAGTGACGGCAAACATGACT	TAGCCAGCGTACCGGATGA	
Cd86	GCACGTCTAAGCAAGGTCACC	TGACATTATCTTGTGATATCTGCATGT	
Arg1	GCCTCGAGGAGGGGTAGAGA	AAAGGAGCTGTCATTAGGGACA	
Il10	CAAATTCATTCATGGCCTTGTAGACA	CCCTCAGGATGCGGCTGA	
IL10	CATCAAGGCGCATGTGAAC	AGATGTCAAACTCACTCATGGCT	
Cd206	AAAAACTGACTGGGCTTCCG	ATTCTTCTCTTGTCTGTTGCCATAA	
NGF	ATGTCCATGTTGTTCTACACT	AAGTCCAGATCCTGAGTGTCT	
NT3	TACGCGGAGCATAAGAGTCAC	GGCACACACACAGGACGTGTC	
NT4	CCTCCCCATCCTCCTCCTTTT	ACTCGCTGGTGCAGTTTCGCT	
BDNF	AACAATAAGGACGCAGACTT	TGCAGTCTTTTTGTCTGCCG	
RN18S	CGGACAGGATTGACAGATTG	CAAATCGCTCCACCAACTAA	
Gapdh	AGGACCAGGTTGTCTCCTGC	ACCCTGTTGCTGTAGCCGT	
GAPDH	GGCAAATTCAACGGCACAGT	AGATGGTGATGGGCTTCCC	

20. Western blot analysis and enzyme linked immunosorbent assay

Eluted proteins of conditioned medium or lysates in naïve hNSPCs, GFP-hNSPCs, and IL-10-hNSPCs were investigated by western blot analysis. Lysates were prepared with homogenization in Tissue Protein

Extraction Reagent (Thermo Fisher Scientific, Inc.) supplemented with protease inhibitors (Sigma-Aldrich), and the homogenates were briefly sonicated to shear the DNA before centrifugation at 4°C for 1 hr at 100,000 *g*. The supernatant was stored as the soluble fraction. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) based on the Bradford assay. Proteins were resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes, which were blocked in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween20 (TBST) for 1 hr. After overnight incubation at 4°C with human NT3 antibodies (Santa Cruz Biotechnology, Dallas, TX, USA), the membranes were washed in TBST for 20 min and incubated at room temperature with peroxidase-conjugated anti-mouse or rabbit antibody (Jackson ImmunoResearch Laboratories) for 1 hr. The blot was washed in TBST for 20 min, treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific), and bands were detected using a LAS 4000 mini (GE Healthcare, Buckinghamshire, UK).

The injured brain tissues were homogenized with Precellys Lysing kits (Bertin technologies, Cat # CK14) and Precellys 24 tissue homogenizer (Bertin technologies) in solution containing PBS and cOmplete ULTRA Tablets protease inhibitor cocktail (Roche Life Science) at a ratio of 500 ul to 200 mg tissue. Samples were centrifuged at 19,000 g for 20 min at room temperature, and then supernatants were collected and stored at -80°C. The concentration of mouse TNF-α and IL-1β and human IL-10 were determined using DuoSet® ELISA Development Systems for mouse TNF-α and IL-1β and human IL-10 (R&D systems) according to the manufacturer's protocol.

21. Statistical analysis

Statistical analyses were conducted with SPSS 20.0 (IBM Company, Somers, NY, USA). On parametric analysis, differences among multiple means with one variable were calculated by one-way ANOVA and the Bonferroni post hoc test, and differences between two means were estimated with

unpaired t-test. On non-parametric analysis, differences among multiple means with one variable were evaluated by Kruskal Wallis, and differences between two means were assessed with Mann-Whitney test. Only values with $P < 0.05$ were accepted as significant.

III. RESULTS

1. Generation and Characterization of IL-10-expressing hNSPCs *in vitro*

IL-10-expressing hNSPCs (IL-10-hNSPCs) and GFP-expressing hNSPCs (GFP-hNSPCs) were generated by infection with lentiviral particles encoding the human IL-10 and GFP, and GFP only, respectively (Fig. 1A). We first characterized GFP-hNSPCs and IL-10-hNSPCs *in vitro* and found that they formed neurospheres and expressed GFP (Fig. 1B). Flow cytometry analysis confirmed that 95.8% of GFP-hNSPCs and 89.8% of IL-10-hNSPCs were GFP positive (Fig. 1C). IL-10 mRNA and protein were strongly expressed only in IL-10-hNSPCs, but not in GFP-hNSPCs (Fig. 1D and E). IL-10 from the supernatants of IL-10-hNSPCs and GFP-hNSPCs was quantified by enzyme linked immunosorbent assay (ELISA). The concentration of IL-10 in IL-10-hNSPCs was 87.4±5.72 ng/ml (1×10^6 cells/2 ml of media/72 hr), whereas IL-10 was not detectable in GFP-hNSPC and hNSPC samples (Fig. 1F).

To compare the proliferation rate, we performed EdU labeling experiments on IL-10-hNSPCs and GFP-hNSPCs. Cells were cultured in N2 media containing mitogens and 2 μM of EdU, which is a thymidine analogue incorporated into the newly synthesized DNA. Twenty-four hr after EdU treatment, IL-10-hNSPCs and GFP-hNSPCs were dissociated into single cells, stained with Alexa Fluor® 647 azide (Invitrogen) and analyzed by flow cytometry (LSR II, BD). IL-10-hNSPCs and GFP-hNSPCs exhibited 33.1±1.39% and 47.4±0.35% EdU positivity among total cells, respectively (Fig. 1G). To confirm this effect of IL-10 on proliferation, hNSPCs were cultured in absence or presence of 100 ng/ml of IL-10 peptide, and then incubated for 24 hr with EdU. The percentage of EdU-positive hNSPCs was significantly increased in IL-10 peptide treated hNSPCs (22.9±0.43%) compared to control hNSPCs (19.6±0.19%) (Fig. 1H).

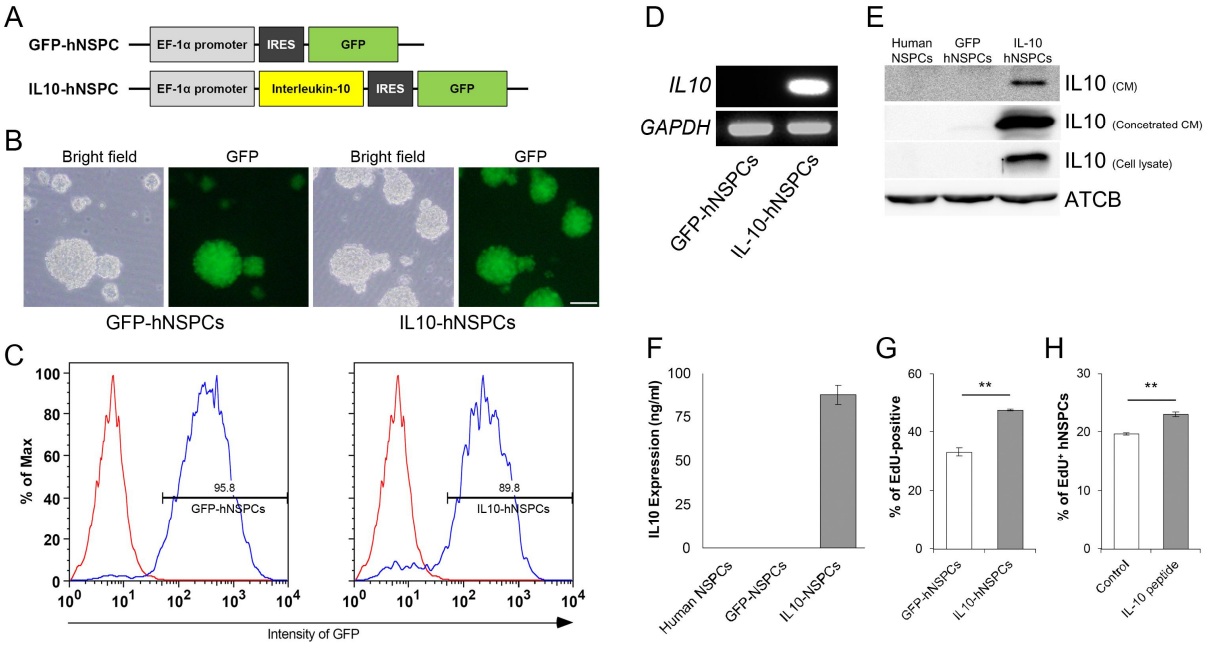


Figure 1. Characterization of IL-10-expressing hNSPCs *in vitro*. (A) Structure of lentiviral vector carrying human interleukin-10 under the control of the EF-1α promoter and GFP as a reporter gene, placed under IRES control. (B) Bright filed and immunofluorescence microscopy of representative neurospheres of IL-10-hNSPCs and GFP-hNSPCs. GFP signal was stably expressed in GFP-hNSPCs and IL-10-hNSPCs. Scale bar=100μm. (C) Flow cytometry; 95.8% of GFP-hNSPCs (blue line histogram, left) and 89.8% of IL-10-hNSPCs (blue line histogram, right) were GFP positive. Non-infected cells (hNSPCs) were used as a negative control (red line histogram). (D) RT-PCR was performed with the cDNA synthesized from mRNA of GFP-hNSPCs and IL-10-hNSPCs. Messenger RNA of IL-10 was only detected in IL-10-hNSPCs, but not in GFP-hNSPCs. (E) Expression of the interleukin-10 protein on Western blot. IL-10 was only detected in conditioned media and cell lysates of IL-10-hNSPCs. (F) ELISA assay performed using the supernatants of cultured hNSPCs, GFP-hNSPCs and IL-10-hNSPCs. (G) EdU-positive hNSPCs were significantly increased in IL-10-hNSPCs compared to GFP-hNSPCs (n=3 per group). (H) EdU-positive hNSPCs were significantly increased in 100 ng/ml of IL-10 peptide-treated hNSPCs compared to hNSPCs without IL-10 peptide treatment (n=3 per group). Data are presented as mean ± standard error of the mean (SEM). **P<0.01 between the two groups.

2. Differentiation of IL-10-hNSPCs and GFP-hNSPCs *in vitro*

To examine the differentiation patterns of GFP-hNSPCs and IL-10-hNSPCs *in vitro*, immunocytochemical staining with various antibodies was performed at 5 days after cell plating in the culture dishes under differentiation conditions. GFP-hNSPCs and IL-10-hNSPCs showed immunoreactivity for the immature cell marker NESTIN (Fig. 2A and B; 91.3±1.95% vs 79.9±1.52%, respectively, n=3), astrocyte markers GFAP (Fig. 2C and D; 81.7±0.71% vs 19.6±2.31%), early neuronal marker TUJ1 (Fig. 2E and F; 14.0±1.82% vs 34.9±4.27%), and oligodendrocyte precursor markers OLIG2 (Fig. 2G and H; 9.8±0.02 vs 5.0±1.56%).

The percentage of TUJ1 in IL-10-hNSPCs was significantly increased, whereas the percentage of NESIN, GFAP, and OLIG2 was significantly decreased in IL-10-hNSPCs versus GFP-hNSPCs (Fig. 2I). Quantitative real-time PCR (RT-PCR) analysis of cultured cells under differentiation conditions confirmed that TUJ1 mRNA level of IL-10-hNSPCs was significantly increased, whereas the mRNA level of GFAP and GALC (oligodendrocyte marker) were significantly decreased in IL-10-hNSPCs (Fig. 2J).

These findings demonstrate that IL-10-expressing hNSPCs show neurogenesis at the expense of astrocyte and oligodendrocyte differentiation *in vitro*.

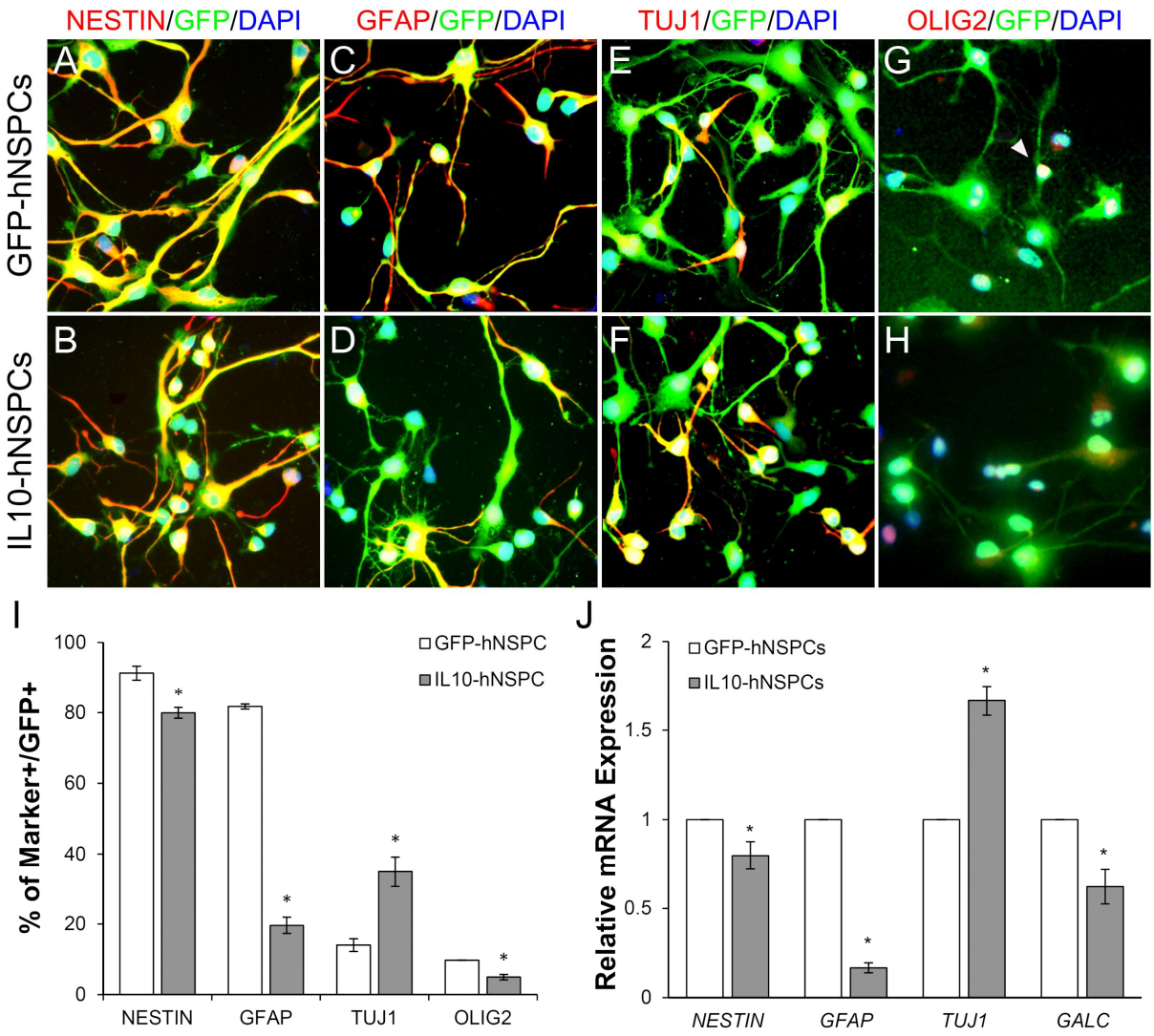


Figure 2. The differentiation patterns of IL-10-hNSPCs and GFP-hNSPCs *in vitro*. (A–H) Representative images of cellular markers in IL-10-hNSPCs and GFP-hNSPCs, Scale bar = 100 μm. Arrow head indicates co-expression of OLIG2 and GFP. (I) Percentages of NESTIN-, GFAP-, TUJ1- and OLIG2-positive cells among total GFP-positive cells were calculated in GFP-hNSPCs and IL-10-hNSPCs under differentiation conditions (n=3 per group). (J) Relative fold change of mRNA expression for NESTIN, GFAP, TUJ1 and GALC, was measured by qRT-PCR analysis under differentiation conditions (n=3 per group). Data are presented as mean ± SEM. *P<0.05 compared to the GFP-hNSPCs group.

3. Engraftment and distribution of IL-10-hNSPCs following transplantation

To evaluate the migration and engraftment pattern of hNSPCs in the injured brain, we transplanted IL-10-hNSPCs and GFP-hNSPCs into the damaged brain of mice exposed to HI at 3 days post injury. Animals were sacrificed and dissected at 2 or 4 wk post transplantation for immunohistochemistry staining to detect grafted human cells with human specific Nuc (hNuc) and/or GFP antibodies. At 2 wk post transplantation, grafted hNSPCs could still be detected around the ischemic boundary zone of the ipsilateral hemisphere and in the external capsule and cortex of the contralateral hemisphere (Fig. 3E–H).

Transplanted cells were co-stained with hNuc and GFP antibodies (Fig. 3B–D). Most GFP-positive cells were present in the ischemic boundary zone (Fig. 3E and F), and a few hNSPCs were infrequently distributed in the external capsule (Fig. 3G) and cortex (Fig. 3H) of the contralateral hemisphere at 2 wk after injection. On the other hand, we could rarely detect GFP-positive cells by immunohistochemical analysis of the lesion area at 4 wk post transplantation. We have identified the expression of human specific IL-10 in IL-10-hNSPCs *iv vivo*, but human IL-10 protein was only observed in IL-10-hNSPCs transplanted mice (Fig. 3I–K show high magnification images, Fig. 3M–O show low magnifications images, and Fig. 3L show the orthogonal view from confocal *z*-series). However, we could not detect human IL-10 protein expression in GFP-hNSPCs *in vivo* (Fig. 3P–R).

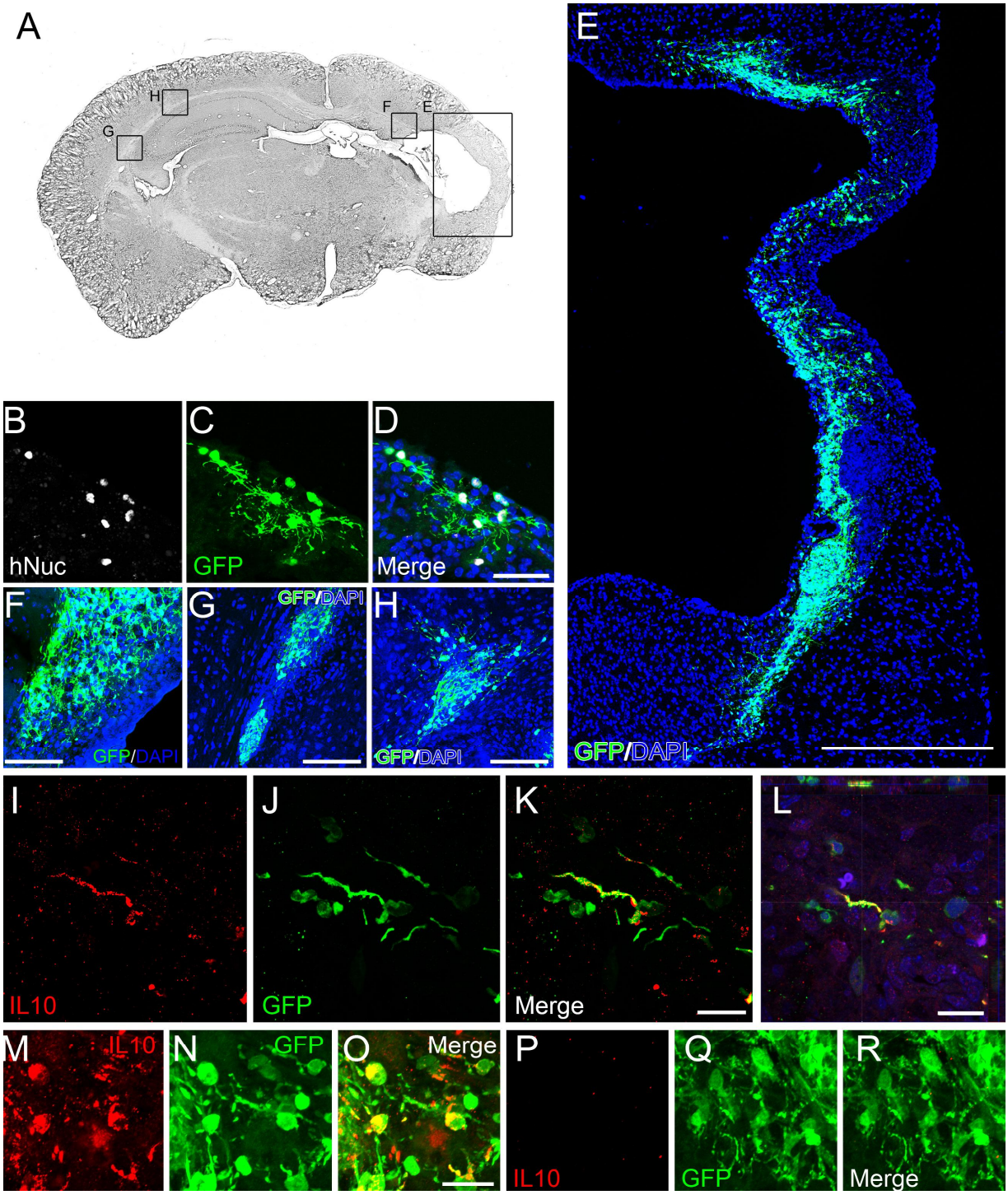
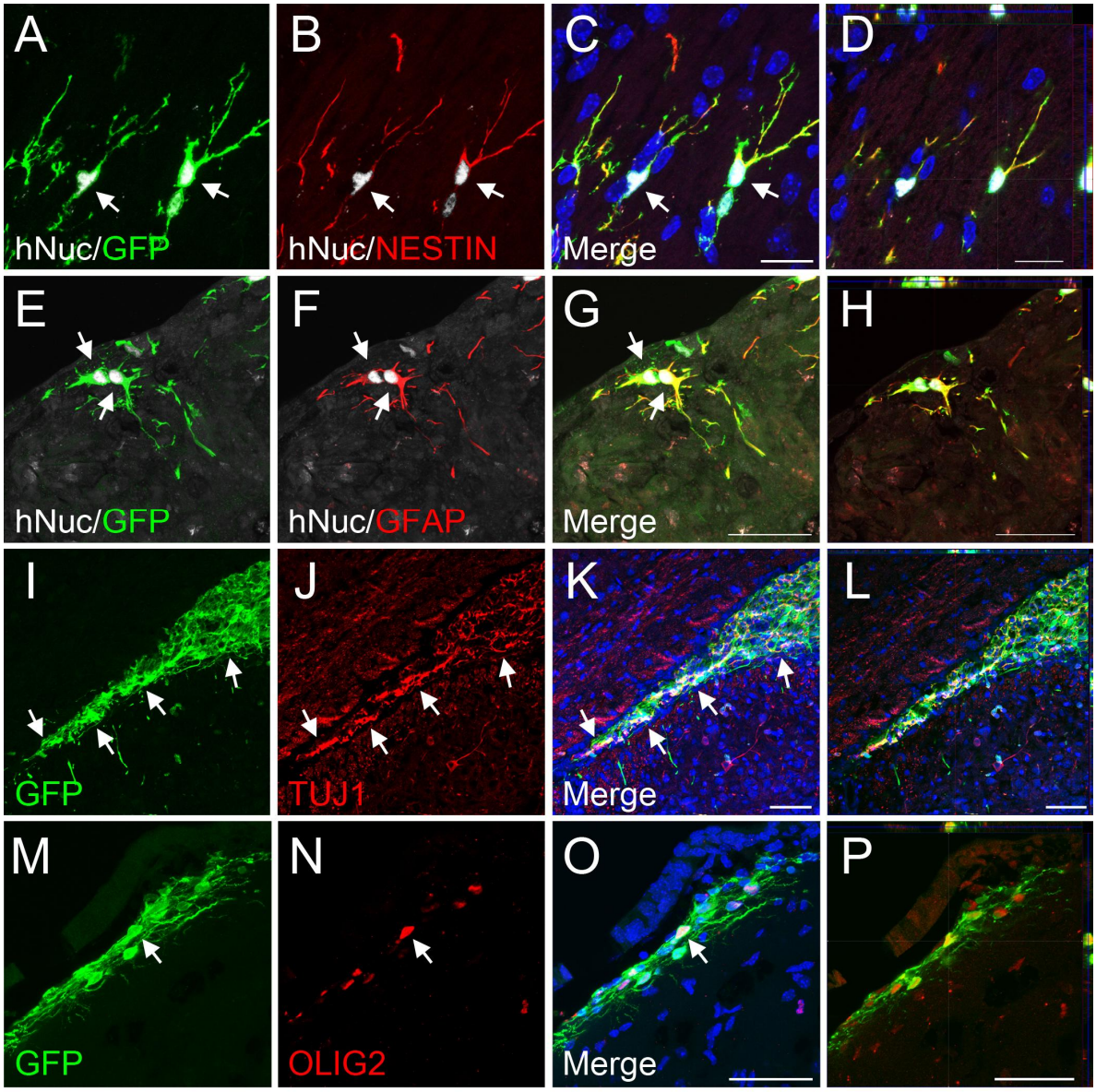


Figure 3. Engraftment and distribution of IL-10-hNSPCs *in vivo*. (A) Low-magnification view of stroke within the mouse brain. The black boxes in Fig. 3A represent regions for representative images at 2 wk post-transplantation. (B–D) Grafted cells into the brain co-expressed human-specific nuclei (hNuc; white)

and GFP (green). (E–G) Most GFP-positive cells were found in the ischemic boundary zone (E) and spared hippocampal region (F), with a few cells infrequently detected in the external capsule (G) and cortex (H) of the contralateral hemisphere at 2 wk post transplantation. (I–K and M–O) Maximum Intensity Projection (MIP) images showed that IL-10-hNSPCs co-expressed human IL-10 (red) and GFP (green): (I–K) high magnification and (M–O) low magnification. (L) Orthogonal view from confocal *z*-series confirmed that GFP (green) and human IL-10 (red) in cytoplasm were expressed in the same cell. (P–R) GFP-hNSPCs did not express human IL-10 *in vivo*. Scale bar=50 μm (D), 200 μm (E), 100 μm (F-H), 20 μm (K and L) and 25 μm (O).

4. Differentiation of IL-10-hNSPCs and GFP-hNSPCs in HI mice following transplantation

We examined the differentiation patterns of IL-10-hNSPCs and GFP-hNSPCs following transplantation into the ischemic lesion site of HI injured mice. In the brain of HI mice that received IL-10-hNSPCs, the grafted cells expressed human NESTIN (78.4±0.47%; Fig.4A–D), GFAP (78.9±3.19%; Fig.4E–H), TUJ1 (45.5±1.85%; Fig. 4I–L) and OLIG2 (3.3±0.72%; Fig.4M–P). Compared to the GFP-hNSPC-transplanted HI mice, a significantly higher number of grafted cells differentiated into TUJ1-positive early neurons by 27.4%, and a lower number of grafted cells expressed human NESTIN by 14.1% in the IL-10-hNSPCs-transplanted group. There was no significant change in the percentages of GFAP positive astrocytes between GFP-hNSPCs and IL-10-hNSPCs groups (Fig. 4R). In both groups, few GFP-positive cells (less than 3%) expressed OLIG2 specific to oligodendrocyte progenitor cells.



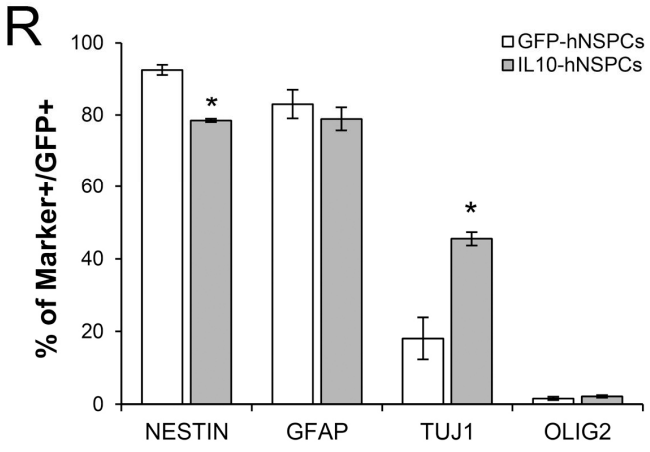


Figure 4. Differentiation of IL-10-hNSPCs in HI mice following transplantation. (A–P) GFP^+ cells (green) co-expressed cell type-specific markers (red), such as NESTIN (A–D), GFAP (E–H), TUJ1 (I–L), and OLIG2 (M–P). White arrows indicate the co-expression of each marker and GFP. (D, H, L, and P) Orthogonal view from confocal *z*-series showed that GFP and cell type-specific markers were expressed in the same cell. Scale bar=20 μm (C and D), 50 μm (G, H, K, L, O and P). (R) Comparison of differentiation patterns between IL-10-hNSPC and GFP-hNSPC transplantation groups. **P*<0.05 compared to GFP-NSC group. All bars represent mean ± SEM.

5. HI mice implanted with IL-10-expressing hNSPCs exhibit enhanced functional recovery

We monitored neurological performance using neurological severity scores (NSS) and the cylinder test at 1, 2, 3, and 4 wk post transplantation. IL-10-hNSPCs injected HI mice (IL10-NSC group) showed significant functional recovery using NSS from 1 through 4 wk post transplantation compared to the H-H buffer injected group (vehicle group) and at 1, 3 and 4 wk post transplantation compared to the GFP-hNSPCs-transplanted group (GFP-NSC). However, there were no significant NSS differences between intact and IL10-NSC groups, and vehicle and GFP-NSC groups at all time points (Fig. 5A).

In the cylinder test, the IL10-NSC group showed improved performance at 3 and 4 wk post transplantation compared to the vehicle group and at 3 wk post transplantation compared to the GFP-NSC group. No significant changes were found between intact and IL10-NSC groups, and vehicle and GFP-NSC groups at all time points (Fig. 5B).

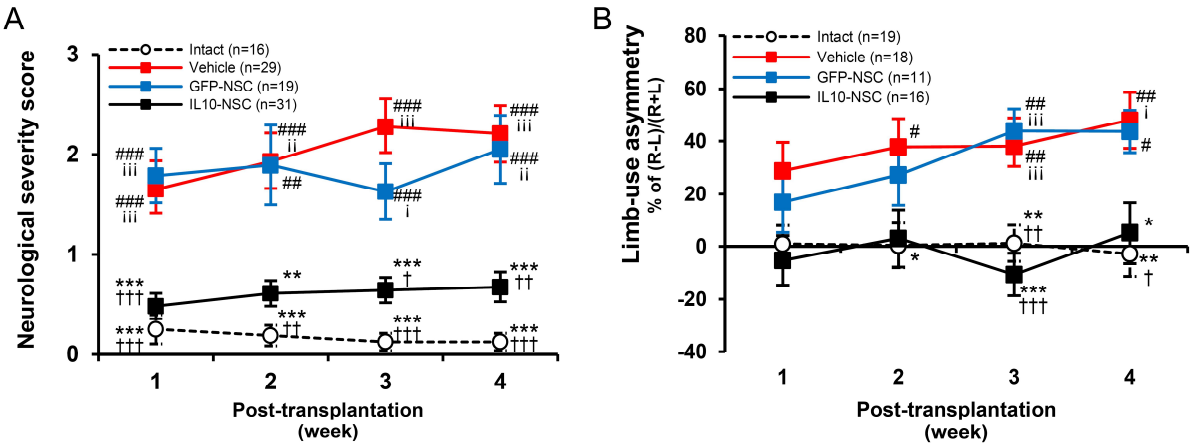


Figure 5. Effect of IL-10-hNSPCs on behavioral performances. IL10-NSC group showed significant differences in neurological severity score (A) and cylinder test (B) compared to the other groups. All data are presented as mean ± SEM. #P<0.05, ##P<0.01, ###P<0.001 compared to the intact group; **P<0.01, ***P<0.001 compared to the vehicle group; $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001 compared to the GFP-NSC group; ¡P<0.05, ¡¡ P<0.01, ¡¡¡P<0.001 compared to the IL10-NSC group.

6. Transplantation of IL-10-hNSPCs reduces infarct size after HI brain injury

We investigated whether transplantation of IL-10-hNSPCs could facilitate amelioration of ischemic stroke. At 4 wk post transplantation, the percentages of infarct volume in the IL10-NSC, GFP-NSC, and vehicle groups were 37.6±4.63%, 50.4±3.95% and 57.6±2.86%, respectively, as determined by haematoxylin and eosin staining (Fig. 6A). Infarct size of the IL10-NSC group was significantly decreased by 20% compared to the vehicle group, but there was no significant change in infarct volume between GFP-NSC and vehicle groups (Fig. 6C).

We performed TUNEL staining to label apoptotic cells in the ischemic boundary zone at 4 wk post transplantation. A significant decrease of TUNEL-positive nuclei was found in the IL10-NSC group (8.4±1.15 nuclei/mm^2) compared to the vehicle and GFP-NSC groups (21.1±2.33 nuclei and 19.3±2.98 nuclei/mm^2, respectively). There was no significant change between vehicle and GFP-NSC group (Fig.

6D).

These results suggest that IL-10-hNSPCs exert a neuroprotective effect *in vivo*.

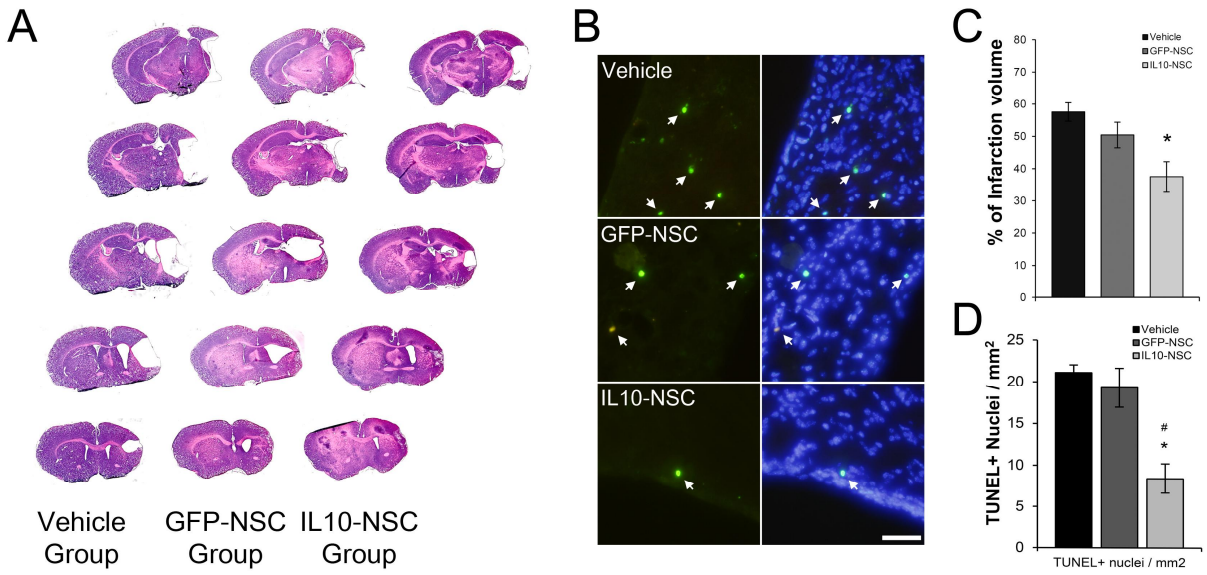


Figure 6. Effect of IL-10-hNSPCs on infarct size. (A) Infarct size measured by haematoxylin and eosin staining at 4 wk post transplantation. (B) TUNEL-positive nuclei in the ischemic boundary zone at 4 wk post transplantation. White arrows indicate TUNEL-positive DAPI. (C) Cortical infarct size was significantly attenuated in the IL10-NSC group compared to the vehicle group (n=6–8 per group). (D) The number of TUNEL-positive nuclei was significantly in the IL10-NSC group compared to the vehicle and GFP-NSC groups (n=4–7 per group). *P<0.05 compared to the vehicle group; #P<0.05 compared to the GFP-NSC group. All bars represent mean ± SEM.

7. Effects of IL-10-expressing human NSPCs on inflammation *in vitro*

Several lines of evidence suggest that neuronal injury related to inflammation can induce a cascade of immune responses that are involved in the pathogenesis of early brain injury.[56-58] Animal models demonstrate several changes in proinflammatory cytokine expression in conjunction with neuronal

damage.[57,59] In these models, ischemia or hypoxia alone is not enough to cause cerebral damage, but additional inflammatory signals contribute to cell death.[60] Therefore, we investigated the effects of IL-10-hNSPCs on modulation of inflammatory responses *in vitro*, such as proinflammatory cytokine expression, proliferation and migration of microglia, and polarization of primary monocyte by FACS.

First, we confirmed the elevated mRNA level of proinflammatory cytokines (TNF-α, IL-1b, IL-6, and iNOS) when BV2 cells were treated with 100 ng/ml of LPS (LPS/Media group) (Fig. 7A). IL-6 and iNOS mRNA of the activated BV2 with GFP-hNSPCs CM (LPS/GFP-CM group) were significantly decreased compared to LPS/Media group, although TNF-α mRNA in the GFP-CM treated group was reduced with a decreased tendency (P=0.052) compared to LPS-treated BV2 cells. There was no change of IL-1b between the LPS/Media and LPS/GFP-CM group. The mRNA of proinflammatory cytokines (TNF-α, IL-1b, IL-6, and iNOS) of the IL-10-hNSPCs CM-treated BV2 (LPS/IL10-CM) group were significantly decreased compared to the LPS/Media group, and TNF-α and IL-1b genes of the LPS/IL10-CM group were more decreased than the LPS/GFP-CM group (Fig. 7A).

We also used RAW264.7 (murine macrophage cell line) to confirm the effect on reduction of inflammatory cytokine expression (Fig. 7B). As BV2 microglial cells, proinflammatory cytokine genes were increased in 100 ng/ml of LPS-treated RAW264.7 cells. The mRNA expression level of TNF-α and IL-6 in the LPS/GFP-CM group was significantly reduced compared to the LPS-treated RAW264.7 cells, but IL-1b and iNOS levels of LPS/GFP-CM group were not changed compared to LPS-treated RAW264.7 with LPS. The gene expression levels of TNF-α, IL-1b and IL-6 in LPS/IL10-CM group were significantly decreased compared to LPS-treated RAW264.7 cells, and those of TNF-α and IL-6 treated with IL-10-hNSPCs CM were more decreased than the LPS/GFP-CM group. There was no significant change of iNOS mRNA level among the LPS/Media, LPS/GFP-CM, and LPS/IL10-CM groups (Fig. 7B).

These results show that hNSPCs have the capacity to inhibit proinflammatory cytokine expression, and this effect was more enhanced by IL-10-hNSPCs (Fig 7A and B).

Next, we quantified proliferation of BV2 co-cultured with fibroblast, GFP-hNSPCs and IL-10-hNSPCs.

Using the Transwell system with 0.4μm pore size, we assessed the effect of secretary factors from IL-10-hNSPCs or GFP-hNSPCs on microglial proliferation. The percentages of EdU-positive BV2, indirectly co-cultured with media only (BV2 group), fibroblast (BV2/Fibroblast) and GFP-hNSPCs (BV2/GFP-NSC), were 71.0±0.26%, 71.2±0.44% and 68.3±1.00%, respectively, showing no significant changes between these groups. However, BV2 with IL-10-hNSPCs (BV2/IL10-NSC) showed a significant decreased effect on proliferation (53.4% ± 2.74%) compared to the other three groups (Fig. 7C). Direct co-cultured BV2 with hNSPCs exhibited dramatic changes in comparison with the indirect co-culture system. The percentages of proliferation of BV2, directly co-cultured with only media, fibroblast, GFP-hNSPCs and IL-10-hNSPCs, were 66.9% ± 0.35%, 43.1% ± 0.15%, 33.0% ± 0.63% and 5.9% ± 0.06%, respectively. Compared to the BV2/Fibroblast group, the percentages of EdU-positive cells were significantly decreased in BV2/GFP-CM and BV2/IL10-CM groups by 10.1% and 37.2%, respectively. The BV2/IL10-CM group was more reduced than BV2/GFP-CM group (Fig. 7D).

These data show that not only secretary factors but also transmembrane proteins of IL-10-hNSPCs influence the proliferation rate of microglia, although we have not identified factors that inhibit proliferation.

Next, we estimated whether GFP-hNSPCs and IL-10-hNSPCs affected migration of BV2 and THP-1 (human monocyte cell line) toward hNSPCs. Fibroblast, GFP-hNSPCs and IL-10-hNPCs were seeded onto 24 well culture dishes at 5×10^5 cells/well without mitogen for 24 h, and then 5×10^4 cells of BV2 were added into 5μm pore Transwell placed on the 24-well dishes. After 4 hours BV2 cell seeding, migratory cells through the Transwell membrane were measured by Cyquant GR dye. Migration of BV2 and THP-1 (human monocyte cell line) toward both hNSPCs was significantly increased, compared to recruitment of those cells toward fibroblast or media, although IL-10 did not enhance or weaken the migratory capacities of BV2 and THP-1 (Fig. 7E and F). These data are consistent with a previous report that shows the chemotaxis of macrophages/monocytes is only marginally impaired by IL-10.[61]

We measured the polarization of macrophages isolated from mouse leg bones (femur and tibia) into

M1 (classically activation) or M2 (alternatively activation) phenotypes. We defined M1 macrophage as MHC class II^+ and $CD206^-$ and M2 macrophage as MHC class II^- and $CD206^+$ among CD11b-positive cells by flow cytometry (Fig. 7G). The percentages of M1 macrophages induced by culture media, GFP-hNSPCs CM and IL-10-hNSPCs CM were 9.4% ± 0.08%, 7.1% ± 0.03% and 3.3% ± 0.12%, respectively. M1 macrophage induction was significantly decreased by CM of GFP-hNSPCs and IL-10-hNSPCs, and the IL-10-hNSPCs CM-treated group were more decreased than the GFP-hNSPCs CM-treated group (Fig 7H). In contrast, M2 macrophage induction was significantly enhanced with GFP-hNSPCs CM (52.5% ± 0.02%) and IL-10-hNSPCs CM (63.7% ± 0.06%) compared to the media only group (48.5% ± 0.07%). Thus, treatment with IL-10-hNSPCs CM had better efficacy on M2 induction than that of GFP-hNSPCs CM (Fig. 7I).

These data demonstrate that IL-10-hNSPCs have several effects on decreased expression of cytokines from BV2 and RAW264.7, inhibited proliferation of murine microglial cell line and polarization of bone marrow derived monocytes into alternative activated macrophages. However, IL-10-hNSPCs does not affect migration of BV2 and THP-1.

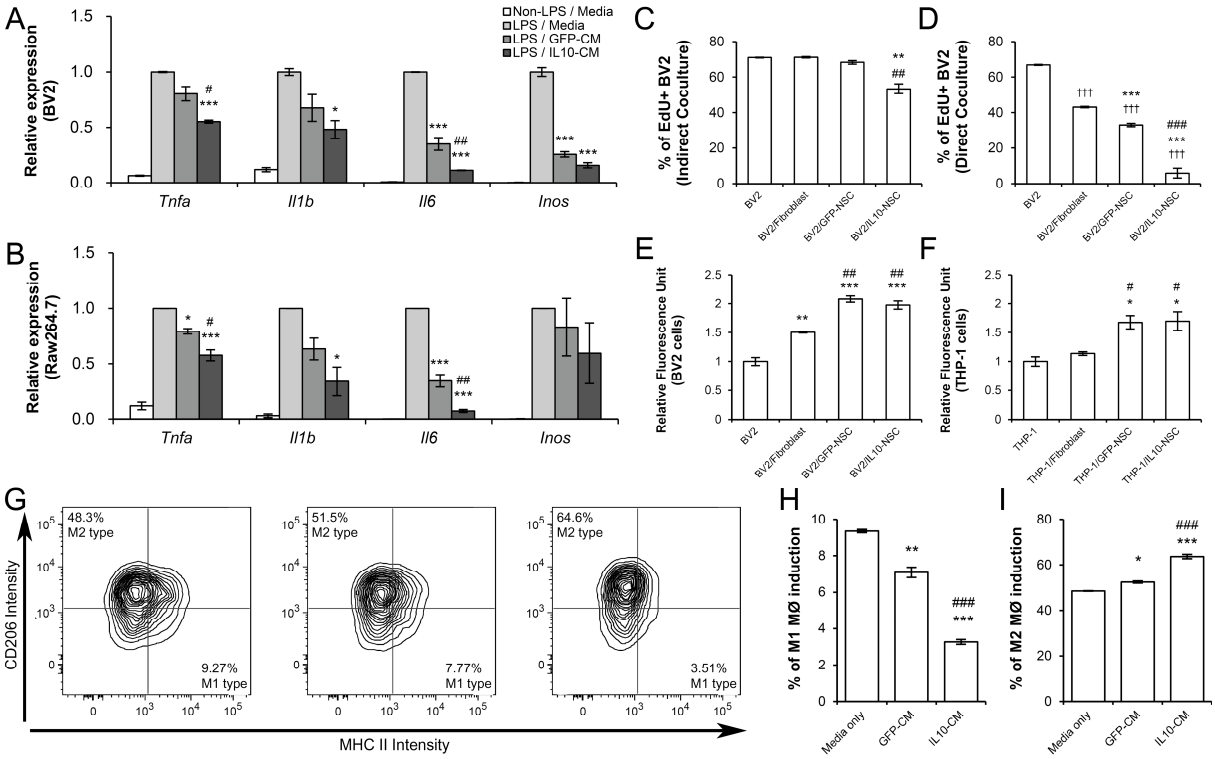


Figure 7. Effects of IL-10-hNSPCs on inflammation *in vitro*. (A, B) IL-10-hNPSCs suppressed LPS-induced TNF-α, IL-1b, IL-6 and iNOS mRNA in BV2 (murine microglial cell line) and RAW264.7 (murine macrophage cell line). The cultures were treated CM from IL-10-hNSPCs (IL10-CM) or GFP-hNSPCs (GFP-CM) supplemented with or without LPS (100 ng/ml) and incubated for 24 hours. Total RNA was extracted and subjected to real-time PCR quantification for expression of proinflammatory mediator genes (n=3 per group). *P<0.05, ***P<0.001 compared to the LPS-treated group; #P<0.05, ##P<0.01 compared to the GFP-CM group. (C, D) The percentage of EdU-positive BV2 in total cells was calculated following treatment with CM from fibroblast, GFP-hNSPCs or IL-10-hNSPCs. (C) In the indirect co-culture by Transwell with 0.4μm pore size, the percentage of EdU-positive BV2 co-cultured with IL-10-hNSPCs (BV2/IL10-NSC) was more decreased than that of fibroblast CM- (BV2/Fibroblast) or GFP-hNSPCs CM-treated BV2 (BV2/GFP-NSC). (D) In the direct co-culture, IL-10-hNSPCs and GFP-hNSPCs reduced the proliferation of BV2 co-cultured with fibroblast. IL-10-hNSPCs had better effect on proliferation compared to the BV2/GFP-NSC group (n=3 per group). ††† P<0.001 compared to

the BV2 group; **P<0.01, ***P<0.001 compared to the BV2/Fibroblast group; ##P<0.01, ###P<0.001 compared to the BV2/GFP-NSC group. (E, F) BV2 or THP-1 (human monocyte cell line) cells, passed through Transwell with 5um pore size, were quantified by Cyquant GR dye, which binds to cellular nucleic acids, produces a large fluorescence and measures cell numbers. Migration of BV2 and THP-1 into GFP-hNSPCs (BV2/GFP-NSC and THP-1/GFP-NSC, respectively) or IL-10-hNSPCs (BV2/IL10-NSC and THP-1/IL10-NSC) were significantly increased, compared to migratory cells into fibroblast (BV2/Fibroblast and THP-1/Fibroblast). There was no significant difference in BV2 or THP-1 between the GFP-NSC and IL10-NSC groups (n=3 per group). *P<0.05, **P<0.01, ***P<0.001 compared to BV2 group; #P<0.05, ##P<0.01 compared to the BV2/Fibroblast group. (G-I) Reduction of M1 macrophage and induction of M2 macrophage were observed when we treated conditioned media from IL-10-hNSPCs (IL10-CM) and GFP-hNSPCs (GFP-CM) into primary macrophages. (G) Representative images of polarization of monocyte into M1 (MHC II-positive and CD206-negative fraction) or M2 macrophage (MHC II-negative and CD206-positive fraction) by flow cytometry. (H, I) These changes were more significant in the IL10-CM group than the GFP-CM group (n=3 per group). All data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared to the media only condition; ###P<0.01 compared to the GFP-CM group.

8. Effects of IL-10-hNSPCs during progression of brain inflammation in HI mice

Proinflammatory mediator expression is enhanced immediately after injury and is followed several days later by a period of enhanced expression of anti-inflammatory cytokines.[62] While the immune response in the days and weeks after brain injury can benefit recovery by clearing cellular debris and producing neurotrophic factors, disproportionate expression of neurotoxic proinflammatory mediators in the first hours and days after injury may be detrimental.[63] Therefore, we examined the expression of cytokines using quantitative real-time PCR for mRNA level and ELISA for protein level at 1 wk post-

transplantation (Fig. 8A and B). Messenger RNA level of IL-1b, IL-6 and iNOS was significantly reduced in IL10-NSC group compared to the vehicle group, and IL-6 gene showed a significant decrease in the IL10-NSC group compared to the GFP-NSC group (Fig. 8A).

The protein level of TNF-α and IL-1β was measure by ELISA. Mouse TNF-α of IL10-NSC group (1.0±0.08 pg/μg) was significantly decreased compared to the GFP-NSC (1.5±0.03 pg/μg) and vehicle (1.7±0.13 pg/μg) groups. Mouse IL-1β (0.15±0.011 pg/μg) was also significantly reduced in the IL10-NSC group compared to the GFP-NSC (0.21±0.010 pg/μg) and vehicle (0.26±0.019 pg/μg) groups (Fig. 8B).

We isolated immune cells from the HI injured brain using Percoll gradient (37%-70%) centrifugation at 1 wk post transplantation. The isolated immune cells were stained with CD11b and CD45 antibodies, analyzed by flow cytometry. Three different populations were defined: $CD11b^{+}/CD45^{low}$, $CD11b^{+}/CD45^{high}$, and $CD11b^{-}/CD45^{+}$ cells. The majority of isolated immune cells were CD11b-positive cells, with most being $CD11b^{+}/CD45^{low}$ and some $CD11b^{+}/CD45^{high}$ (Fig. 8C and D). $CD11b^{+}/CD45^{low}$ cells were significantly increased in the IL10-NSC group (75.2±9.89%) compared to the GFP-NSC group (34.3±9.69%), and $CD11b^{+}/CD45^{high}$ cells were decreased in the IL10-NSC group (7.1±3.21%) compared to the GFP-NSC group (21.7±6.20%) with almost formal significance (P=0.108). There were no significant differences in $CD11b^{+}/CD45^{low}$ and $CD11b^{+}/CD45^{high}$ cells percentage between the IL10-NSC and vehicle groups.

Using magnetic beads conjugated with CD11b antibodies, we specifically isolated CD11b-positive cells from the injured brain and analyzed for M1 (TNF-α) or M2 (Arginase 1 (Arg1), IL-10 and CD206) marker genes by real-time PCR. Messenger RNA level of TNF-α was significantly increased in the IL10-NSC group compared to the GFP-NSC group, but there was no significant difference between the IL10-NSC and vehicle groups. Compared to the vehicle group, Arg1 was significantly increased in both IL10-NSC and GFP-NSC groups, but CD206 was significantly enhanced in only the IL10-NSC group. Messenger RNA of IL-10 expressed in $CD11b^{+}$ cells from IL10-NSC group was significantly increased

compared to vehicle and GFP-NSC groups (Fig. 8E).

To confirm these *in vivo* results, we co-cultured BV2 directly with media, GFP-hNSPCs or IL-10-hNSPCs *in vitro*. In these *in vitro* data, CD86, a marker of classical activation,[16,64] was not changed among all groups. Compared to the media only condition (BV2 co-cultured with media), Arg1 mRNA expression level of GFP-NSC (BV2 with GFP-hNSPCs) and IL10-NSC (BV2 with IL-10-hNSPCs) groups were significantly increased more than 340 and 160 fold, respectively, although Arg1 was significantly decreased in the IL10-NSC group compared to the GFP-NSC group. Other markers of alternative activation, such as IL-10 and CD206, were significantly enhanced in the IL10-NSC group compared to the GFP-NSC group (Fig. 8F).

These data show that IL-10-hNSPCs inhibit gene expression and protein production of proinflammatory cytokines, deactivate the classically activated microglia, and augment mRNA expression of the alternative activation markers (Arg1, IL-10 and CD206) *in vivo*.

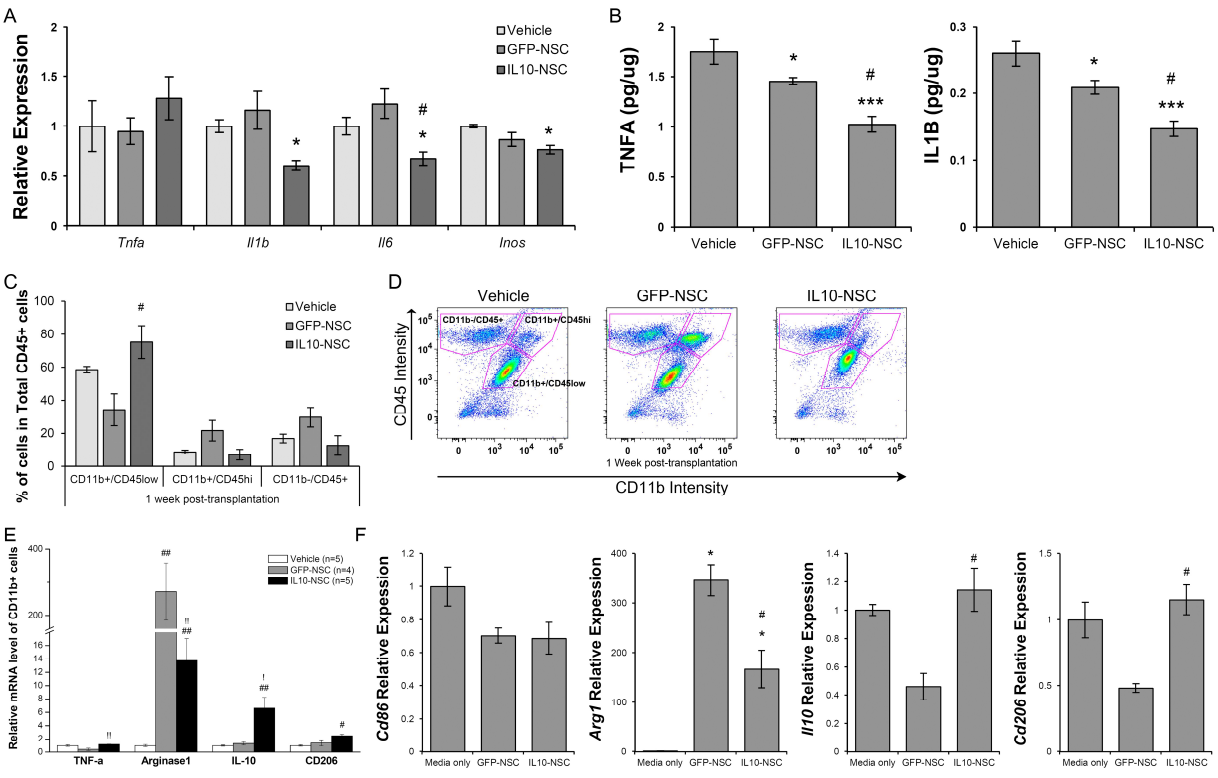


Figure 8. Effects of IL-10-hNSPCs on modulation of inflammatory responses at 1 wk post transplantation. (A) Relative expression level of mouse cytokine mRNA by quantitative real-time PCR. (B) Quantification of mouse TNF-α and IL-1β proteins by ELISA in the ipsilateral hemisphere of HI brain at 1 wk post transplantation (n=3–4 for quantitative real-time PCR and n=6–7 for ELISA per group). *P<0.05, ***P<0.001 compared to the vehicle group; #P<0.05 compared to the GFP-NSC group. (C) The percentage of $CD11b^{+}/CD45^{low}$, $CD11b^{+}/CD45^{high}$, and $CD11b^{-}/CD45^{+}$ among total CD45-positive cells isolated from the HI brain at 1 wk post transplantation (n=4–5 per group). $CD11b^{+}/CD45^{low}$ population of the IL10-NSC group was significantly increased compared to the GFP-NSC group. There were no significant differences for $CD11b^{+}/CD45^{high}$ and $CD11b^{-}/CD45^{+}$ among all groups. #P<0.05 compared to the GFP-NSC group. (D) Representative flow cytometry for $CD11b^{+}/CD45^{low}$, $CD11b^{+}/CD45^{high}$, and $CD11b^{-}/CD45^{+}$ in the HI brain of vehicle, GFP-NSC, and IL10-NSC groups at 1 wk post transplantation. (E) Phenotype of isolated CD11b-positive cells from the injured hemisphere at 1 wk post transplantation was determined by quantitative real-time PCR analysis of M1 marker TNF-α, and M2 markers Arg1, IL-10, and CD206 (n=4–5 per group). #P<0.05, ##P<0.01 compared to the vehicle group; !P<0.05, !!P<0.01 compared to the GFP-NSC group. (F) *In vitro* microglial phenotype of BV2 co-cultured with media, GFP-hNSPCs, or IL-10-hNSPCs directly was assessed by quantitative real-time PCR analysis of M1 marker CD86, and M2 markers Arg1, IL-10, and CD206 (n=3 per group). *P<0.05, ***P<0.001 compared to the media only group; #P<0.05 compared to the GFP-NSC group All data are presented as mean ± SEM.

9. Effects of IL-10-hNSPCs on brain inflammation at late stage of HI brain injury

We analyzed the occupied area by Iba-1 in both hemispheres of HI mice at 4 wk post transplantation with H-H buffer, GFP-hNSPCs and IL-10-hNSPCs (vehicle, GFP-NSC, and IL10-NSC group, respectively). Representative images of Iba-1-positive cells (Fig. 9B) were captured in the boxes near the ischemic boundary zone of the ipsilateral hemisphere (a, b and c in Fig. 9A). The Iba-1-immunoreactive

area was significantly increased in both GFP-NSC and IL10-NSC groups compared to the vehicle group, and there was no significant change between the GFP-NSC and IL10-NSC groups (Fig 9B and D).

We stained with CD68 antibody to detect activated microglia/macrophages. The area occupied by CD68 for the GFP-NSC group was significantly increased in the ipsilateral hemisphere ($P<0.05$) and slightly increased in the contralateral hemisphere ($P=0.081$) compared to the vehicle group. In both sides of the brain, $CD68^+$ area was significantly reduced in the IL10-NSC group compared to the GFP-NSC group. There was no significant change between the vehicle and IL10-NSC groups on both hemispheres (Fig. 9C and D).

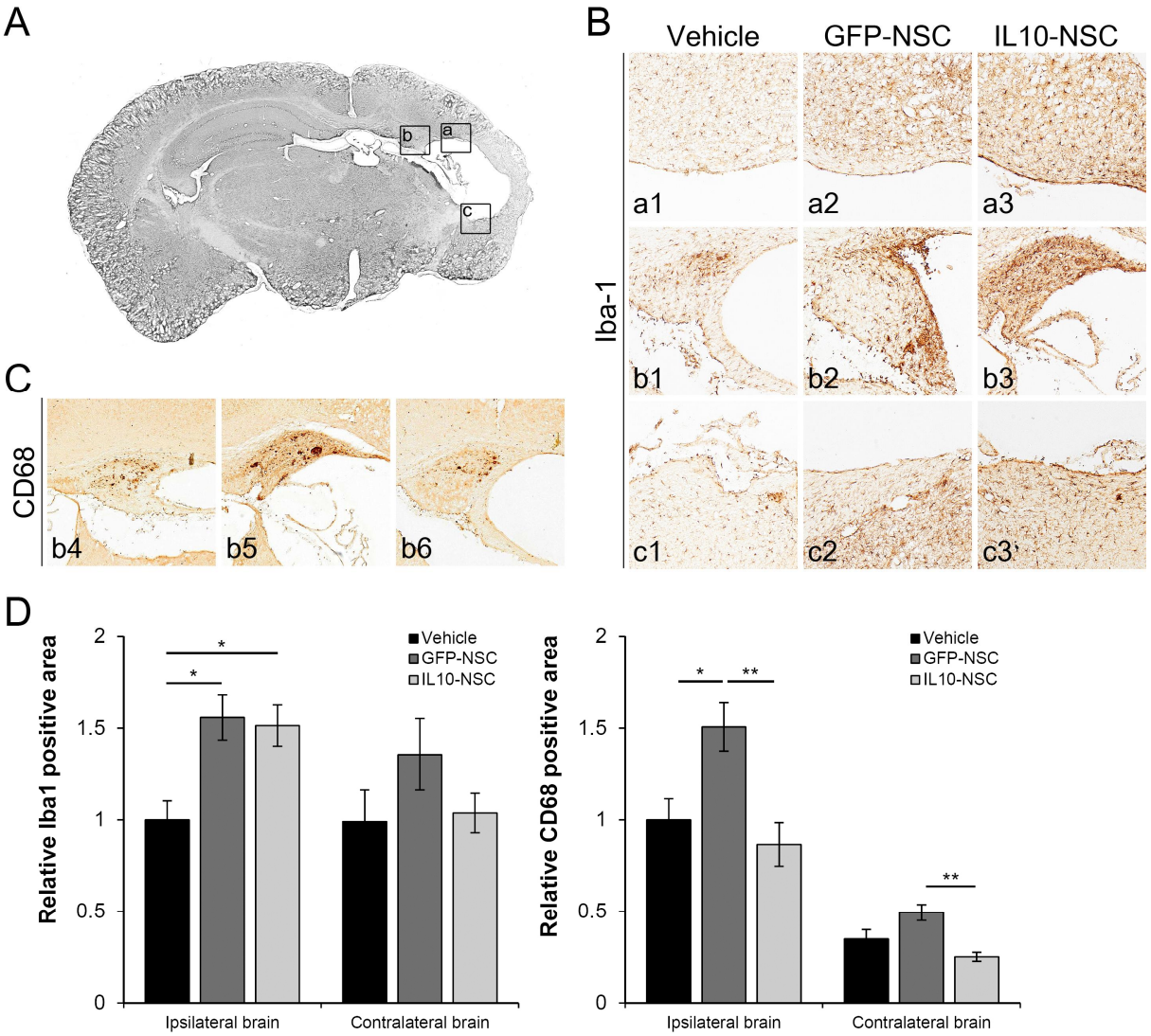


Figure 9. Effects of IL-10-hNSPCs on inflammation at late stage of HI brain injury. (A) A low-magnification view of stroke within the mouse brain. The three black boxes in Fig. 9A represent the regions for representative images at 4 wk post transplantation. (B) Representative images of Iba-1-positive cells for total microglia/macrophages near the ischemic boundary zone. (C) Representative images of CD68-positive cells for activated microglia/macrophages near the ischemic boundary zone. (D) The area occupied by Iba-1-immunoreactive microglia in the ipsilateral hemisphere of HI mice was significantly increased the IL10-NSC and GFP-NSC groups compared to the vehicle group. There was no change in area occupied by Iba-1-positive cells in the ipsilateral hemisphere between IL10-NSC and GFP-

NSC groups. In the contralateral hemisphere of HI mice, there were no significant differences among vehicle, GFP-NSC and IL10-NSC groups. The area occupied by CD68-positive activated microglia in the ipsilateral hemisphere was significantly increased in the GFP-NSC group compared to the vehicle group, and significantly decreased in the IL10-NSC group compared to the GFP-NSC group. There was no significant difference between the vehicle and IL10-NSC groups. In the contralateral hemisphere of the HI brain, the CD68-positive area in the GFP-NSC group showed a strong increasing trend toward significance (P=0.081) compared to the vehicle group. There was a significant decrease of CD68-immunoreactive area in the IL10-NSC group compared to the GFP-NSC group, but no significant differences between the vehicle and IL10-NSC groups (n=7–11 per group). *P<0.05 and **P<0.01 compared between the groups. All data are presented as mean ± SEM.

10. Effects of IL-10-expressing human NSPCs on neuroprotection *in vitro*

To address the neuroprotective effect of IL-10-hNSPCs and GFP-hNSPCs, LDH cytotoxicity assays were performed with differentiated-SH-SY5Y cells treated with retinoic acid. Human neuroblastoma SH-SY5Y, originally derived from the SK-N-SH cell line,[65] have been frequently used, either in their undifferentiated state,[66-68] or in a neuron-like differentiated state after induction with all-trans-retinoic acid.[69-72] SH-SY5Y cells were seeded onto 24-well dishes at 8×10^4 cells/well with 10 μM of retinoic acid and incubated in a humidified incubator at 37°C and 5% CO_2 in air for 5 days. Old media was replaced with fresh serum-free media and 0.4μm pore sized-Transwells were placed on the 24-well dishes. Media or 8×10^4 cells of fibroblast, GFP-hNSPCs and IL-10-hNSPCs were added into the upper chamber of the Transwell for 24 hours. The Transwells were then removed, and SH-SY5Y cells co-cultured with media, fibroblast, GFP-hNSPCs, or IL-10-hNSPCs (SH-SY5Y, SH-SY5Y/Fibroblast, SH-SY5Y/GFP-NSC, and SH-SY5Y/IL10-NSC groups, respectively) were incubated under normoxic conditions in DMEM with glucose (non-OGD) or under anoxic conditions in DMEM without glucose (OGD) for 4 hours. SH-

SY5Y/IL10-NSC group cells showed reduced cytotoxicity, compared to SH-SY5Y/Fibroblast and SH-SY5Y/GFP-NSC groups under non-OGD condition and to the SH-SY5Y/Fibroblast group under OGD condition (Fig. 10A).

We, then, assessed the expression level of neuroprotection-associated genes of IL-10-hNSPCs and GFP-hNSPCs by qRT-PCR. The mRNA expression level of NGF, NT3, NT4 and BDNF were significantly increased in IL-10-hNSPCs compared to GFP-hNSPCs (Fig. 10B).

These data also suggest that IL-10-hNSPCs exerts neuroprotective effects by secretary factors, such as NGF, NT3, NT4 and BDNF *in vitro*.

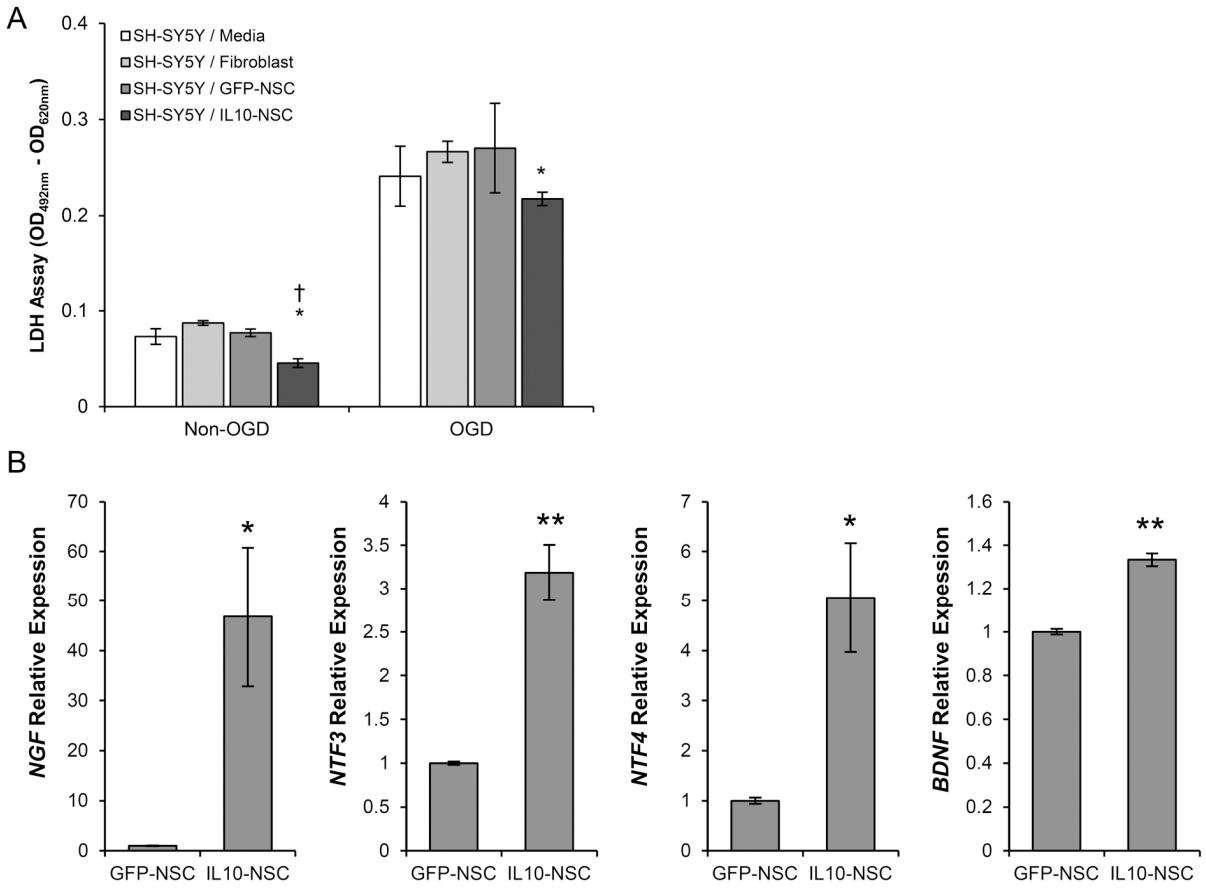


Figure 10. Neuroprotective effects of IL-10-hNSPCs *in vitro*. (A) Viability of SH-SY5Y cells co-cultured with fibroblast, GFP-hNSPCs and IL-10-hNSPCs (SH-SY5Y/Fibroblast, SH-SY5Y/GFP-NSC and SH-

SY5Y/IL10-NSC groups, respectively) were assessed by measuring the LDH activity in the supernatants. Decreased cytotoxicity of the SH-SY5Y/IL10-NSC group only was observed, compared to the SH-SY5Y/Fibroblast and SH-SY5Y/GFP-NSC groups under normoxic conditions, and to the SH-SY5Y/Fibroblast group under OGD conditions (n=3 per group). *P<0.05 compared to the SH-SY5Y/Fibroblast group; †P<0.05 compared to the SH-SY5Y/GFP-NSC group. (B) Relative expression level of NGF, NT3, NT4 and BDNF mRNAs from GFP-hNSPCs and IL-10-hNSPCs was quantified by qRT-PCR (n=3 per group). *P<0.05, **P<0.01 compared to the GFP-NSC group. All bars represent mean ± SEM.

11. Effects of IL-10-expressing human NSPCs on angiogenesis *in vitro*

We performed cell migration assay of endothelial progenitor cells to investigate the angiogenic role of hNSPCs *in vitro*. Chemotactic migration of EPCs was dose-dependently enhanced by IL-10-hNSPCs CM, and it was more potent than that of GFP-hNSPCs CM (Fig. 11A). EPCs treated with vascular endothelial growth factor (VEGF) were used as a positive control in cell migration assay. To clarify candidates that induce angiogenesis, we assessed the expression level of angiogenesis and tissue remodeling-associated genes of IL-10-hNSPCs and GFP-hNSPCs by RT-PCR. The mRNA expression level of MMP1, MMP2, and PAI-1 were increased in IL-10-hNSPCs compared to GFP-hNSPCs (Fig. 11B).

These data also suggest that IL-10-hNSPCs exerts angiogenic effects by secretary factors *in vitro*, such as MMP1, MMP2 and PAI-1.

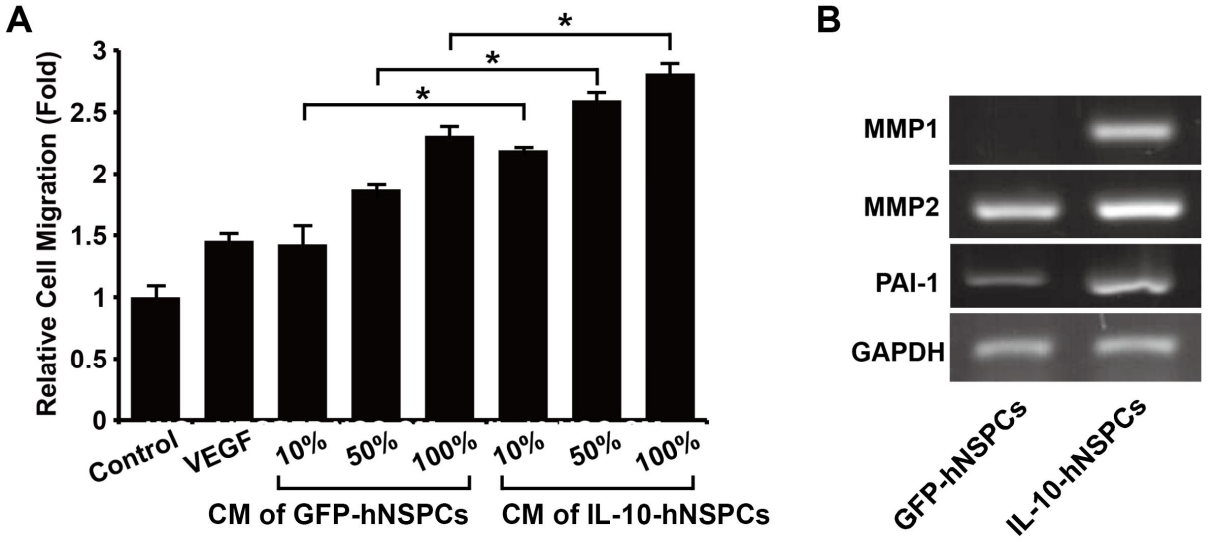


Figure 11. Angiogenesis effects of IL-10-hNSPCs *in vitro*. (A) Dose-dependent effects of GFP-hNSPCs CM and IL-10-hNSPCs CM on chemotactic migration of EPCs. Migratory cells were quantified under microscopy at × 100 magnifications after staining with hematoxylin and eosin (n=4 per group). (B) Relative expression level of MMP1, MMP2, and PAI-1 mRNAs from GFP-hNSPCs and IL-10-hNSPCs was observed by RT-PCR. *P<0.05 compared between the groups. All data are presented as mean ± SEM.

IV. DISCUSSION

Microglial activation is the initial step in inflammatory responses of the CNS to various stimuli, such as stroke[73] and hypoxic-ischemic brain injury.[74] This initial step amplifies the inflammatory response in the ischemic brain. The microglial population is heterogeneous, and a subpopulation of microglia protects against stroke: injury is exacerbated after depletion of the proliferative microglia[75] and ameliorated after injection of microglia into the ischemic brain.[76] In other neurological disorders, appropriately activated microglia may also protect neurons from damage induced by resident or infiltrating cells, such as spinal cord injury,[19,77-79] traumatic brain injury[80-82] and stroke.[83-85] Previous studies have shown that transplantation of neural stem cells can modulate cerebral inflammation and protect the brain from degeneration.[38,45,46,86] However, transplantation of neural stem cells in neonatal HI brain is not sufficient to decrease the infarct volume, although these cells recover functional impairment.[87-90] Several studies have suggested that neural stem cells combined with other treatments, such as chondroitinase ABC[89] or mild hypothermia,[90] are more effective against HI brain injury in terms of both improved neurological function and reduced infarct volume, but not when neural stem cells are administered alone. Therefore, we hypothesized that hNSPCs combined with human IL-10 would attenuate HI brain injury in immature mice by modulating inflammation causing the secondary injury.

To confer a strong anti-inflammatory capacity to hNSPCs, we transduced these cells with IL-10 using lentiviral vectors. Human NSPCs, genetically modified to express IL-10, release it stably, until the cells are diminished *in vivo*. IL-10-hNSPCs can be specifically located and express a transgene to locally modulate inflammation in the damaged site, because of their homing properties toward brain injuries.[91,92] We also showed that transplantation of IL-10-expressing hNSPCs after neonatal HI brain injury can be very effective. IL-10-hNSPCs transplantation reduced lesion size significantly and markedly improved functional outcome.

We established IL-10-hNSPCs infected by lentiviral vectors encoding human IL-10, and characterized

these cells *in vitro* using Western blot, ELISA, quantitative real time-PCR, immunocytochemistry and flow cytometry. While most hNSPCs transduced by IL-10- or GFP-encoded lentiviral vectors stably expressed GFP, IL-10 was only expressed in IL-10-hNSPCs, evaluated by RT-PCR, Western blot and ELISA.

We measured the proliferation rate of IL-10-hNSPCs and GFP-hNSPCs. The EdU uptake was significantly enhanced in IL-10-hNSPCs. Interleukin-10-expressing hNSPCs were affected by IL-10 released from themselves, acting in both an autocrine and paracrine manner. To confirm the effect of IL-10 on hNSPCs proliferation, we treated these cells with IL-10 peptide, and the percentage of EdU-positive hNSPCs was significantly increased. These data show that IL-10 can affect hNSPCs proliferation. Although we have not demonstrated the precise molecular mechanism of IL-10 in promoting the proliferation of hNSPCs, several studies have shown that IL-10 affects proliferation of neural stem cells *in vitro* and increases the population of progenitors in the subventricular zone *in vivo*.[93-95] Pereira et al.[95] demonstrated that Erk signaling was activated by IL-10 in neural progenitor cells, and it had been previously reported that activating phosphorylation of Erk1/2 promotes proliferation of neural stem cells.[96] Thus, we can infer that hNSPCs proliferation is induced via activation of the Erk1/2 signaling pathway by IL-10.

While hNSPCs possess the potential to differentiate into neurons, IL-10-hNSPCs differentiate into more neurons than GFP-hNSPCs, both *in vitro* and *in vivo*. The mechanism underlying IL-10-induced neural cell differentiation is not clear; it is possibly by induction of neurotrophic factors such as BDNF and NT-3. IL-10 production has been positively correlated to BDNF and NT-3 production, which promote neuronal proliferation, survival, and differentiation,[97,98] and reduce astrogliosis.[99] We also found that BDNF and NT-3, as well as NGF and NT-4, were induced in IL-10-hNSPCs cultures. The astrocyte marker, GFAP, was significantly decreased in IL-10-hNSPCs compared to GFP-hNSPCs *in vitro*, whereas there was no difference in the percentages of GFAP-positivity between IL-10-hNSPCs and GFP-hNSPCs in the ischemic brain *in vivo*. Transplantation studies in various neurodegenerative disease

models support that the extent of neuronal or glial differentiation of hNSPCs depends on both local microenvironmental signals of host brain and intrinsic programs of the grafted cells.[100,101] There are many other variables that may be involved in the differentiation of grafted hNSCs in injured brain, such as growth and inflammatory factors that regulate neurogenesis in the developing and adult brain.[102] Astrocyte differentiation of neural stem/progenitor cells is enhanced by TNF-α and IL-1β *in vitro* and brain inflammation related to neurological diseases.[103-105] This discrepancy of astrocyte differentiation between *in vitro* and *in vivo* is probably due to the inflammation of the HI brain.

In terms of differentiation *in vitro* and *in vivo*, the sum of all quantification markers of hNSPCs often reported more than 100%, suggesting there was overlap between some markers.[41,106,107] In fact, nestin has been found to co-localize with OLIG2, TUJ1 and/or GFAP. In addition, several studies have observed differentiation failure of engrafted hNSPCs following transplantation.[39,40,108-111] Thus, these findings mean that some of the grafted cells in this study express both neuronal (TUJ1) and glial (GFAP) cell markers suggesting their immature state *in vitro* and *in vivo*.

Our data show that interleukin-10-expressing hNSPCs have superior anti-inflammatory effects than GFP-hNSPCs on activated microglia and macrophages *in vitro*. The expression level of proinflammatory cytokines mRNA from BV2 and RAW264.7 cells was more decreased by treatment with IL-10-hNSPCs conditioned media. The supernatants of IL-10-hNSPCs significantly reduced the proliferation rate of microglia, and IL-10-hNSPCs resulted in more decreased proliferation of microglia when microglia were directly co-cultured with GFP-hNSPCs or IL-10-hNSPCs. Our previous data show that not only secretory factors but also hNSPCs transmembrane molecules are related to microglial deactivation, and these effects in particular are more enhanced by cell-to-cell contact.[106] CD200 and CD47 constitutively maintain microglia in a resting state and impede their proinflammatory activity.[106,112]

We also assessed the impact of IL-10-hNSPCs on monocyte/macrophage polarization *in vitro*. Enhanced M2 and impaired M1 type polarization were observed in IL-10-hNSPCs CM-treated bone marrow derived macrophages. According to the other reports, M2c type macrophages are known as

deactivated or regulatory macrophages[113] and are elicited by IL-10 and TGF-β; M2c macrophages appear to be involved in tissue remodeling.[114-116] We have previously identified the expression of TGF-β mRNA from hNSPCs.[106] Therefore, we propose that IL-10-hNSPCs enhance M2 polarization of bone marrow derived macrophages by IL-10 and TGF-β *in vitro*.

Using an *in vivo* model of neonatal HI brain injury, we verified the results from the *in vitro* study. Ivacko et al.[117] demonstrated that activated microglia begin to accumulate within 4 hours after injury and accumulation of lectin-positive cells peak at 2–4 days post injury in perinatal rats, although they did not distinguish microglia and macrophages from lectin-positive cells. Appropriately activated microglia and macrophages at sub-acute injury stage may also protect neurons from damage induced by resident or infiltrating cells. Therefore, we transplanted IL-10-hNSPCs to regulate microglial activation at 3 days post injury, and analyzed inflammation and microglia/macrophages activation at 7 days post transplantation *in vivo*. To assess the modulating effects of IL-10-hNSPCs on inflammation in the ischemic brain *in vivo*, the brains were dissected to quantify cytokine levels by real-time PCR and ELISA, as well as polarization of microglia and/or macrophages by real-time PCR and flow cytometry at 7 days post transplantation. Protein and gene levels of IL-1β were reduced in IL-10-hNSPCs-injected mice compared to GFP-hNSPCs-injected mice. TNF-α protein level was significantly decreased in the IL10-NSC group compared to the vehicle and GFP-NSC groups, although the gene level of TNF-α did not change significantly among the group. The correlation between mRNA and protein quantity depends on various biological and technical factors,[118] and these constraints make the correlation between mRNA and protein worse.

Numerous experimental studies have highlighted depicted the pivotal response of resident microglia and infiltrating monocyte-derived macrophages to either the development of the brain injury or its resolution leading to conflicting interpretation of their protective or deleterious contribution in stroke.[119,120] In neonates, however, the infiltration of neutrophils, lymphocytes and macrophages following ischemia and focal stroke may be less profound[121-123] or only briefly present in the

parenchyma.[124] Several lines of evidence support that not only microglia can be distinguished from macrophages and other immune cells, but also resting microglia can be discriminated from activating microglia using CD11b and CD45 membrane antigens.[125-129] To date, the most common characterization of resting microglia and activating microglia/infiltrating macrophages are $CD11b^{+}/CD45^{low}$ and $CD11b^{+}/CD45^{high}$, respectively, using flow cytometry.[126,128-131] Compared to adult stroke, infiltration of circulating macrophages/monocytes across the blood brain barrier is relatively low during the acute injury phase in neonates.[121-123] It remains poorly understood whether leukocyte immaturity at the time of insult or a distinct gene expression pattern of selectins, and cytokines/chemokines account for the difference. The issue regarding infiltration of macrophages and monocytes into neonatal ischemic brain remains unresolved.[86,123,132-134]

Therefore, we supposed that $CD11b^{+}$ cells were resting microglia and activated microglia/infiltrating macrophages at sub-acute stage injury stage in the HI brain of neonates. We identified 3 different populations by flow cytometry with CD11b and CD45 antibodies: $CD11b^{+}/CD45^{low}$ (resting microglia), $CD11b^{+}/CD45^{high}$ (activating microglia/infiltrating macrophages) and $CD11b^{-}/CD45^{+}$ (other infiltrating cells). Our flow cytometry data show that microglial activation and infiltrating of macrophages in the ischemic brain were inhibited by IL-10-hNSPCs at 1 wk post transplantation, whereas GFP-hNSPCs could not deactivate microglial activation or inhibit infiltration of macrophages in the injured brain.

To identify the phenotype of microglia/macrophages, we isolated $CD11b^{+}$ cells in the brain by real-time PCR, using CD11b antibodies conjugated with magnet beads, and compared differential gene expression levels of M1 and M2 microglia markers among vehicle, GFP-NSC and IL10-NSC groups. Intriguingly, M2 markers, such as Arg-1, IL-10 and CD206, were dramatically increased in microglia/macrophages isolated from the IL10-NSC group compared to the vehicle group. None of IL-10 and CD206 mRNA was changed between vehicle and GFP-NSC groups, but Arg-1 was increased in the GFP-hNSPCs group compared with the vehicle group.

To confirm these *in vivo* results, we cultured BV2 cells with GFP-hNSPCs, IL-10-hNSPCs or culture

media. Our *in vitro* data showed that the expression levels of IL-10 and CD206 were significantly enhanced in microglial cells co-cultured with IL-10-hNSPCs. Although decreased in the IL-10-hNSPCs group compared to the GFP-hNSPCs, arginase-1 gene level was induced in both IL-10-hNSPCs and GFP-hNSPCs group compared to BV2 cells without hNSPCs.

Taken together, these results indicate that IL-10-hNSPCs regulate protein production and gene expression levels of cytokines via modulating polarization of microglia toward alternative activation or deactivation *in vivo*, and these anti-inflammatory effects are more enhanced in IL-10-expressiong hNSPCs than GFP-hNSPCs at the sub-acute stage of injury.

We evaluated whether the modulatory effects of IL-10-hNSPCs on inflammation of the ischemic brain were sustained until sacrifice. Using immunohistochemistry with Iba-1 antibody, the area occupied with microglia in the HI brain was quantified at 4 wk post transplantation. Contrary to our expectation based on the increase of resting microglia by IL-10-hNSPCs at 1 wk post transplantation, the area occupied by Iba-1-positive microglia was significantly increased in both GFP-NSC and IL10-NSC groups compared to the vehicle group. These data are consistent with previous studies that showed an increase in microglia or microglial activation in the neonatal ischemic or healthy brain that received neural stem cells (NSCs) or human NSCs.[86,88,135]

To investigate whether these microglia were deactivated at 4 wk post transplantation, we used CD68 antibody to detect activated microglia. CD68 is widely used as a microglia/macrophages marker for activation or M1 polarization,[64,136] although no previous reports describe the existence of a single, specific marker for microglia, macrophage or activation status of these cells.[137-139] IL-10-hNSPCs effectively deactivated microglial activation as indicated by measuring the area occupied by CD68-positive cells compared to the GFP-NSC group, but not to the vehicle group. Our staining data using Iba-1 and CD68 antibodies showed that IL-10-hNSPCs effectively deactivated activated microglia, although the increased area occupied by microglia is seen in both IL-10-hNSPCs- and GFP-hNSPCs-transplanted groups.

Transplantation of NSPCs can not only reduce conventional inflammatory responses, but also improve

neuroprotection by secretary factors in various neurological diseases.[97-99,140-142] Neurotrophic factors are important for the survival, maintenance, and regeneration of specific neuronal populations in the brain.[143,144] A decrease in neurotrophic factors has been associated with the pathology of several neurodegenerative diseases and their physiological symptoms.[145,146] Various studies have demonstrated that neurotrophic factors can protect neurons against a variety of pathological insults, including ischemia[147,148] and neurotoxins, such as Alzheimer's disease.[149-151] Angiogenesis is related to neurogenesis and plays a critical role in injured tissue repair.[152] It is important that intracerebral transplantation of hNSPCs can induce angiogenesis, because angiogenesis is implicated in the formation of neurovascular units and functional recovery after ischemic stroke and thus may significantly contribute to a favorable clinical outcome for stroke patients.[153] Angiogenesis involves the migration and proliferation of pre-existing, fully differentiated endothelial cells.[154] In addition, circulating EPCs may home to sites of neovascularization and differentiate into endothelial cells *in situ*.[155-160] Mobilization of EPCs augments neovascularization of ischemic tissue[155,160-163] and may be clinically relevant in the setting of tissue ischemia.[162,164,165]

In addition to the anti-inflammatory properties of IL-10-hNSPCs, we identified that not only the neuroprotective effects on neuron-like SH-SY5Y cells but also the angiogenic effects on endothelial progenitor cells were enhanced by the secreted factors from IL-10-hNSPCs *in vitro*. Although the therapeutic potential of neurotrophic and angiogenic factors was not directly identified in the ischemic brain, many publications suggested that neurotrophic[166-169] and angiogenic factors[153,162,164,165] ameliorate neonatal HI brain injury. Therefore, we cannot rule out the possibility that the therapeutic effects in ameliorating ischemic brain injury are associated with neurotrophic and angiogenic factors secreted from IL-10-hNSPCs.

Transplantation of IL-10-hNSPCs in neonatal HI mice produced improvement of functional recovery as determined by NSS and the cylinder test, and reduction of infarct volume. In the IL-10-hNSPCs-transplanted group, neurological function assessed by NSS test, was significantly recovered during the

observation period, and the forelimb-use asymmetry evaluated by cylinder test was significantly improved at 3 and 4 wk post transplantation compared to vehicle and GFP-NSC groups. The delayed recovery effect apparent for the cylinder test, unlike the NSS test, is due to features of the test. Zarruk et al.[170] note that the cylinder test showed decreased sensitivity in mice with small infarcts, particularly within a month after ischemia. The reduced brain infarct volume was only observed for the IL10-NSC group compared to the vehicle group, although there was no significant difference between the GFP-NSC and IL10-NSC groups. Although transplanted hNSPCs were rarely detected in the injured brain at 4 wk post transplantation, reduced cerebral infarct size and improved behavioral functions were observed until sacrifice. Our data are consistent with other studies that demonstrate the benefit of cell transplantation may not be due to cell replacement, and long term engraftment is not required for stem cells to exert long term function.[25,171]

IL-10 is a pluripotent cytokine with potent effects on numerous cell populations, in particular circulating and resident immune cells as well as epithelial and neuronal cells. Inadequate IL-10 expression seems to have considerable pathophysiological impact. Both overexpression (e.g., in lymphoma, SLE, intensive care unit patients) as well as deficiency (e.g., in inflammatory bowel disease, psoriasis, stroke) of IL-10 are likely to have a pathophysiological significance.[172] Therefore, interleukin-10 may exhibit double-edged sword properties depending on diseases. Transgenic mice had a VMD2 gene promoter that chronically expressed IL-10 at a much higher level than transgenic negative mice.[173] The magnitude depended on the organ tissue. There was a 2-3-fold increase in the sciatic nerve correlating with demyelination caused by infiltrating monocyte-derived macrophages, and a 5-fold increase in the spinal cord dorsal roots. In the CNS, while there was a 100-fold increase of IL-10 expression in the brain, there were no inflammatory cells, demyelination, and degradation of neural tissue in the CNS in general. However, the sciatic nerve demyelination, induced by long term expression of IL-10, caused hind limb weakness and eventually paralysis in 3-mo old mice.[173] This study indicated that high levels of IL-10 released for a chronic period of time may have detrimental peripheral neuropathy but

not necessarily on the CNS, such as the brain and spinal cord. Therefore, chronic administration or overexpression of IL-10 do not seems to pose harm locally in the CNS. However, it is still unknown whether long term exposure to IL-10 influences neurological development and causes side effects to the CNS of humans. It has become increasingly clear that appropriate expression of IL-10 may be the key to how IL-10 not only contributes to the delicate balance between pro- and anti-inflammatory reactions, but also ameliorates hypoxic-ischemic brain injury. Therefore, the precise control of transgene expression is desirable for many basic investigations and therapeutic applications. The expression of transgenes can be regulated with more sophisticated methods, such as viral vectors under control of the doxycycline-regulated promoter.[174-176] For clinical applications, further investigations are needed to understand for the long term treatment of IL-10 to the CNS and/or circulatory system, and well-designed human clinical trials are necessary to confirm the long term safety and efficacy of IL-10 for the treatment of hypoxic-ischemic brain injury and other neurological diseases.

We postulated that these therapeutic effects of IL-10-hNSPCs on behavioral recovery and infarct volume could be related to alternative activation or deactivation of microglia in the ischemic brain, as well as neuroprotection and angiogenesis by secretary factors released from hNSPCs. From the first week post transplantation, resting microglia and/or deactivated microglia were increased in IL-10-hNSPCs-transplanted mice, and these effects were sustained until sacrifice. Therefore, an important function of transplanted IL-10-hNSPCs might be their ability to secrete not only IL-10 but also other neurotrophic and angiogenic factors that modulate inflammation and/or the microenvironment in the ischemic brain to enhance the endogenous tissue repair process.

Our results clearly show the therapeutic potential of IL-10-hNSPC treatment after neonatal HI brain injury for improved long term behavioral function as well as effective reduction of lesion volume. The therapeutic effects of IL-10-hNSPCs were associated with increased expression of neurotrophic and angiogenic factors, and conferred anti-inflammatory properties enhanced by IL-10.

The current study was limited in its focus on mainly inflammation of the injured brain; however, we

have demonstrated that intraventricular transplantation of IL-10-hNSPCs promotes immune modulation in the acute phase of hypoxic-ischemic brain damage in mice. Further studies are required to investigate the more specific mechanisms underlying IL-10-hNSPCs-induced functional recovery after hypoxic-ischemic injury.

V. CONCLUSION

Transplantation of stem cells or their derivatives, as well as the activation of endogenous stem cells within the brain, have been previously proposed as future therapies for neurological diseases, particularly HI brain injury. However, it may seem unrealistic to induce functional recovery only by replacing lost cells and/or protecting existing ones in neonatal brain injury. Other strategies to cure diseases are needed for the future.

In the present study, IL-10 expressing cells (IL-10-hNSPCs) were generated by lentiviral vector. IL-10-hNSPCs not only reduced the expression of proinflammatory cytokines from immune cells and proliferation of microglial cell lines, but also promoted microglia/macrophage polarization toward M2 cells both *in vitro* and *in vivo*. We also verified the neuroprotective effects of elevated secreted factors, such as NGF, NT3, NT4 and BDNF, and angiogenic effects by MMP1, MMP2 and PAI-1 elevated in IL-10-hNSPCs. The animal model for HI brain injury was induced by ligation of the right common carotid artery and incubated in a hypoxic atmosphere of 8% O_2 and 92% N_2 on postnatal day 7. IL-10-hNSPCs were transplanted into the ipsilateral HI-injured cerebral cortex at 3 days post injury (postnatal day 10). Implantation of IL-10-hNSPCs into HI mice significantly improved behavioral recovery and neuroprotective effect on infarct volume, as well as inhibited microglial activation, as measured by the area occupied with CD68-positive cells *in vivo*.

IL-10-hNSPCs also prevented polarization of microglia/macrophages into classically activated cells and promoted alternative activation of microglia/macrophages both *in vitro* and *in vivo*.

Therefore, IL-10-hNSPCs exhibited therapeutic potentials for the management of neonatal HI brain injury through multiple neuroprotective, angiogenic and anti-inflammatory mechanisms.

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ABSTRACT (IN KOREAN)

신생아 저산소성-허혈성 뇌손상 동물모델에서 interleukin-10 발현하는 인간 신경줄기/전구세포를 이용한 뇌 이식

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정광수

신생아 저산소성-허혈성 뇌손상(neonatal hypoxic-ischemic brain injury)은 주산기 가사 (perinatal asphyxia)에 의해서 신생아에서 발병하는 대표적 중증 신경계질환으로 만삭아 1000명 출생아 중 2명에서 발병하고, 약 60%에서 신경발달 장애소견을 보인다. 현재까지의 연구에 의하면 손상된 중추신경계의 재생은 거의 불가능하거나 극히 제한적이기 때문에 대부분의 치료는 합병증을 예방하거나 비정상적인 운동 패턴과 경직을 감소시키는데 중점을 두어왔다. 특히 저산소성-허혈성 뇌손상은 일차손상에 의해 수반되는 염증반응 혹은 싸이토카인 발현으로 인해 뇌 손상을 유래할 수 있다고 알려져 있지만, 그 자세한 기전은 자세히 알려져 있지 않다. 신경줄기세포는 자가갱신(self-renew)하고, 신경원세포 및 신경교세포로 분화할 수 있는 분화의 다능성(multipotency)을 보이는 미성숙 신경 세포이다. 시험관 내에서 증식되어 생체 내 이식이 가능한 신경줄기세포는 숙주 신경계에 이주, 생착, 통합되어 치료적으로 유용한 물질을 분비하고,세포구조학적 및 기능적으로도 적절한 신경세포로 분화 가능하기에 난치성 신경계 질환의 치료에 있어 잠재력을 가지고 있다.

Interleukin-10 (IL-10)은 최초에는 싸이토카인 합성을 억제하는 인자로 소개되었고 향후에 항염증 효과를 갖는 것이 알려졌고, 이후에 IL-10으로 명명이 되었다. 거의 모든 면역세포에서 발현/분비하여 대부분의 면역세포에 작용하여 염증·면역반응을 조절하는 것으로 알려져 있다. 특히 IL-10은 염증성 미세아교세포/대식세포를 항염증성세포로 변화시킨다고 알려져 있으며, 직접 염증반응을 조절하여 염증성 장염(enterocolitis), 다발성경화증(multiple sclerosis), 췌장염(pancreatitis), 당뇨병(diabetes), 내독소혈증(endotoxemia), 관절염(arthritis), 전신성 홍반성 루푸스(systemic lupus erythematosus)와 같은 많은 질병에서 병적 증상의 호전을 가져온다고 보고되어 있다.

본 연구에서는 IL-10을 발현하는 인간 신경줄기/전구세포를 확립하여, 신생아 저산소성 허혈성 뇌 손상 동물모델에 이식하여 세포치료 가능성을 연구하고자 하였다. 그 결과 IL-10 발현 인간 신경줄기/전구세포는 뇌 손상 부위에 이식된 후 뇌병변 부위와 그 주변 부위로 이주하면서 생착함을 보였다. IL-10 발현 인간 신경줄기/전구세포가 이식된 실험군은 대조군에 비해 신경학적 기능검사와 실린더 검사에서 유의한 개선된 효과를 보였다. 또한 대조동물에 비해 실험 동물의 뇌 세포 사멸이 유의하게 감소하였고, 뇌 경색의 크기 역시 효과적으로 감소하는 것을 확인 할 수 있었다. IL-10 발현 인간 신경줄기/전구세포는 대조군 인간 신경줄기/전구세포와 비교하여 신경영양인자(nerve growth factor, neurotrophin 3, neurotrophin 4 및 brain-derived neurotrophic factor) 및 혈관생성인자(MMP1, MMP2, PAI1)들의 발현이 크게 증가하였고, 저산소성 허혈성 조건 하에서 신경세포(SH-SY5Y cells)의 생존율이 유의하게 개선되었다. 또한 IL-10 발현 인간 신경줄기/전구세포 뇌 이식 시 손상된 뇌 미세환경의 변화를 관찰하였는데, IL-10 발현 인간 신경줄기/전구세포는 손상된 뇌 조직에서 염증성 싸이토카인의 발현과 활성화된 미세아교세포/대식세포의 증식을 억제하였고, M2형 미세아교세포/대식세포로의 분화를 촉진함을 시험관 및 뇌 손상 조직에서 확인하였다.

따라서 향후 IL-10 발현 인간 신경줄기/전구세포는 상기의 다양한 기전으로 신생아 저산소성 허혈성 뇌 손상 치료에 새로운 세포유전자 치료법으로 활용될 수 있음을 제시하였다.

핵심되는 말 : 신생아 저산소성 허혈성 뇌손상, 인간 신경줄기/전구세포, interleukin-10, 세포이식, 염증반응

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